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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (c).

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TITLE OF THE INVENTION (500 characters max)

CARCINOEMBRYONIC ANTIGEN FUSIONS AND USES THEREOF

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TITLE OF THE INVENTION
CARCINOEMBRYONIC ANTIGEN FUSIONS AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates generally to the therapy of cancer. More specifically, the present invention relates to polynucleotides encoding fusion proteins wherein the fusion proteins comprise at least a portion of the tumor associated polypeptide carcinoembryonic antigen. The present invention also provides recombinant vectors and hosts comprising said polynucleotides, purified fusion proteins and methods for enhancing an immune response against CEA using the compositions and molecules disclosed herein.

BACKGROUND OF THE INVENTION

The immunoglobulin superfamily (IgSF) consists of numerous genes that code for proteins with diverse functions, one of which is intercellular adhesion. IgSF proteins contain at least one Ig-related domain that is important for maintaining proper intermolecular binding interactions. Because such interactions are necessary to the diverse biological functions of the IgSF members, disruption or aberrant expression of many IgSF adhesion molecules has been correlated with many human diseases.

The carcinoembryonic antigen (CEA) belongs to a subfamily of the Ig superfamily consisting of cell surface glycoproteins. Members of the CEA subfamily are known as CEA-related cell adhesion molecules (CEACAMs). In recent scientific literature, the CEA gene has been renamed CEACAM5, although the nomenclature for the protein remains CEA. Functionally, CEACAMs have been shown to act as both homotypic and heterotypic intercellular adhesion molecules (Benchimol et al., *Cell* 57: 327-334 (1989)). In addition to cell adhesion, CEA inhibits cell death resulting from detachment of cells from the extracellular matrix and can contribute to cellular transformation associated with certain proto-oncogenes such as *Bcl2* and *C-Myc* (*see* Berinstein, *J. Clin Oncol.* 20(8): 2197-2207 (2002)). Sequences coding for human CEA have been cloned and characterized (U.S. Patent No. 5,274,087; U.S. Patent No 5,571,710; and U.S. Patent No 5,843,761. *See also* Beauchemin et al., *Mol. Cell. Biol.* 7:3221-3230 (1987); Zimmerman et al., *Proc. Natl. Acad. Sci. USA* 84:920-924 (1987); Thompson et al. *Proc. Natl. Acad. Sci. USA* 84(9):2965-69 (1987)).

Normal expression of CEA has been detected during fetal development and in adult colonic mucosa. CEA overexpression was first detected in human colon tumors over thirty years ago (Gold and Freedman, *J. Exp. Med.* 121:439-462 (1965)) and has since been found in nearly all colorectal tumors. Additionally, CEA overexpression is detectable in a high percentage of adenocarcinomas of the pancreas, liver, breast, ovary, cervix, and lung. Because of its prevalence in these tumor types and limited normal tissue expression, CEA is considered a self tumor-associated antigen and a target for active and passive immunotherapy. Recent clinical data have established that different vaccine strategies

can generate human B and T cells specific for CEA, providing additional evidence that CEA is a target for molecular and immunological intervention for treatment of these cancer types.

Therapeutic approaches targeting CEA include the use of anti-CEA antibodies (*see* Chester et al., *Cancer Chemother. Pharmacol.* 46 (Suppl): S8-S12 (2000)), as well as CEA-based vaccines (for review, *see* Berinstein, *supra*). The development and commercialization of many vaccines have been hindered by difficulties associated with obtaining high expression levels of exogenous genes. Success of DNA-based vaccines has also been hindered by an inability to generate an immune response of sufficient magnitude in treated individuals. DNA vaccines targeting various proteins have been developed, but the immune responses induced to date by DNA vaccines have been relatively weak compared with conventional vaccines.

The ease of DNA manipulation has offered an opportunity to develop gene fusion vaccine strategies in which antigens are linked to various immunoenhancing elements. Enhancement of immune response to target antigens has been demonstrated in animal models by vectors encoding antigens fused to heat shock protein (HSP) 70 (Liu et al., *J. Virol.* 74: 2888-94 (2000); Cheng et al. *J. Immunol.* 166: 6218-26 (2001); Chen et al., *Cancer Res.* 60: 1035-42 (2000)), to Fc portion of IgG1 (You et al., *J. Immunol.* 165: 4581-92 (2000)), to lysosome-associated membrane protein (LAMP) (Su et al., *Cancer Res.* 62: 5041-48 (2002)), and universal Th epitope from tetanus toxin (Renard et al., *J. Immunol.* 171:1588-95 (2003); King et al., *Nature Med.* 4: 1281-86 (1998); Lund et al., *Cancer Gene Ther.* 10: 365-76 (2003); Padua et al., *Nature Med.* 9(11): 1413-17 (2003); Savelyeva et al., *Nature Biotechnol.* 19: 760-64 (2001); Wahren et al., WO 2004/092216). The enhancement of immune responses to target antigens is particularly relevant for cancer vaccines in view of the limited immunogenicity of tumor antigens and of the need to overcome tolerance to exert effective antitumor effects.

Therefore, despite the identification of the wild-type nucleotide sequences encoding CEA proteins described above, it would be highly desirable to develop a vaccine which is capable of eliciting an enhanced CEA-specific immune response relative to a wild-type full-length CEA cDNA, when delivered to a mammal. It would also be desirable to develop methods for treating or preventing CEA-associated cancers which utilize nucleic acid molecules or proteins that safely and effectively potentiate a CEA-specific immune response.

SUMMARY OF THE INVENTION

The present invention provides polynucleotides encoding fusion proteins wherein the fusion proteins comprise at least a portion of the tumor associated polypeptide carcinoembryonic antigen, fused to a substantial portion of an immunoenhancing element. In preferred embodiments, the CEA portion of the encoded CEA fusion protein is deleted of its C-terminal anchoring domain. In other preferred embodiments, the immunoenhancing element is the minimized domain of *tetanus* toxin

fragment C (DOM), or substantial portion thereof. The present invention also provides recombinant vectors, including but not limited to, adenovirus and plasmid vectors, comprising said polynucleotides and host cells comprising said recombinant vectors. Also provided herein are purified fusion proteins encoded by invention polynucleotides.

The present invention further provides methods for inhibiting the development of a cancer in a mammal, or treating or minimizing an existing cancer, by eliciting an immune response to CEA, such methods comprising administering a vaccine or pharmaceutical composition comprising a CEA fusion protein or a nucleotide sequence encoding a CEA fusion protein, as described herein. In preferred embodiments of the methods herein, the immune response is enhanced relative to the response elicited by a wild-type CEA.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

As used throughout the specification and appended claims, the following definitions and abbreviations apply:

The term "promoter" refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers".

The term "cassette" refers to a nucleotide or gene sequence that is to be expressed from a vector, for example, the nucleotide or gene sequence encoding an hCEA-DOM fusion protein. In general, a cassette comprises a gene sequence that can be inserted into a vector, which in some embodiments, provides regulatory sequences for expressing the nucleotide or gene sequence. In other embodiments, the nucleotide or gene sequence provides the regulatory sequences for its expression. In further embodiments, the vector provides some regulatory sequences and the nucleotide or gene sequence provides other regulatory sequences. For example, the vector can provide a promoter for transcribing the nucleotide or gene sequence and the nucleotide or gene sequence provides a transcription termination sequence. The regulatory sequences that can be provided by the vector include, but are not limited to, enhancers, transcription termination sequences, splice acceptor and donor sequences, introns, ribosome binding sequences, and poly(A) addition sequences.

The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus), bacteriophages and cosmids.

The term "first generation," as used in reference to adenoviral vectors, describes adenoviral vectors that are replication-defective. First generation adenovirus vectors typically have a deleted or inactivated E1 gene region, and preferably have a deleted or inactivated E3 gene region.

The designation "pV1J/hCEAopt" refers to a plasmid construct, disclosed herein, comprising the CMV immediate-early (IE) promoter with intron A, a full-length codon-optimized human CEA gene, bovine growth hormone-derived polyadenylation and transcriptional termination sequences, and a minimal pUC backbone (see EXAMPLE 1). The designation "pV1J/hCEA" refers to a construct essentially as described above, except the construct comprises a wild-type human CEA gene instead of a codon-optimized human CEA gene.

The designation "pV1J/hCEA-DOM" refers to a plasmid construct, disclosed herein, comprising the CMV immediate-early (IE) promoter with intron A, a human CEA gene devoid of its GPI anchor coding sequence, fused at its C-terminal end to the N-terminal domain of Fragment C of tetanus toxoid (DOM), bovine growth hormone-derived polyadenylation and transcriptional termination sequences, and a minimal pUC backbone (EXAMPLE 1).

The designation "pV1J/hCEAopt-LTBopt" refers to a construct essentially as described immediately above, except the construct comprises a codon-optimized human CEA gene devoid of its GPI anchor coding sequence instead of the corresponding portion of the wild-type human CEA gene, fused at its C-terminal end to a nucleotide sequence encoding DOM that has also been codon-optimized for high-level expression in human cells..

The designation "pV1J/rhCEAopt-DOMopt" refers to a construct essentially as described above except that the human codon-optimized CEA gene is replaced with a rhesus monkey CEA gene, codon-optimized for high-level expression in human cells.

The designation "pV1J/hCEA-FcIgG" refers to a plasmid construct, disclosed herein, comprising the CMV immediate-early (IE) promoter with intron A, a human CEA gene devoid of the GPI anchor coding sequence, fused at its C-terminal end to the heavy fragment of constant chain of immunoglobulin G1, bovine growth hormone-derived polyadenylation and transcriptional termination sequences, and a minimal pUC backbone. (EXAMPLE 1). pV1J/hCEAopt-FcIgGopt refers to a construct essentially as described, except the nucleotide sequences encoding CEA and FcIgG have been codon-optimized for high-level expression in human cells.

The designations "Ad5/hCEAopt" and "Ad5/hCEA" refer to two constructs, disclosed herein, which comprise an Ad5 adenoviral genome deleted of the E1 and E3 regions. In the "Ad5/hCEAopt" construct, the E1 region is replaced by a codon-optimized human CEA gene in an E1 parallel orientation under the control of a human CMV promoter without intron A, followed by a bovine growth hormone polyadenylation signal. The "Ad5/hCEA" construct is essentially as described above, except the E1 region of the Ad5 genome is replaced with a wild-type human CEA sequence. The designation "Ad5/hCEAopt-DOMopt" refers to an Ad5 construct, essentially as described above, except that the codon-optimized human CEA sequence is devoid of the GPI anchor coding sequence and is fused at its C-terminus to a codon-optimized nucleotide sequence encoding DOM. Construction of adenovirus vectors comprising various CEA fusions is described in EXAMPLE 2.

The abbreviation "DOM" refers generally to the N-terminal domain of fragment C of *tetanus* toxoid.

As used herein, a "fusion protein" refers to a protein having at least two polypeptides covalently linked in which one polypeptide comes from one protein sequence or domain and the other polypeptide comes from a second protein sequence or domain. The fusion proteins of the present invention comprise a CEA polypeptide or fragment or variant thereof, and a second polypeptide, which comprises a substantial portion of an immunoenhancing element, which, in some cases, is a bacterial toxin. The CEA polypeptide, fragment or variant thereof may be a human CEA or CEA homolog from another species. The polypeptides that comprise the fusion protein are preferably linked N-terminus to C-terminus. The CEA polypeptide and the toxin subunit can be fused in any order. In some embodiments of this invention, the C-terminus of the CEA polypeptide is fused to the N-terminus of the toxin subunit, as exemplified in FIGURE 1A. However, fusion proteins in which the immunoenhancing element is fused to the N-terminus of the CEA polypeptide are also contemplated. The term "CEA fusion protein" is intended to be a general term which refers to a fusion as described above, which comprises a CEA polypeptide or fragment or variant thereof fused to a polypeptide comprising an immunoenhancing element.

The term "CEA-DOM fusion" refers to a nucleic acid sequence in which at least a portion of the CEA gene is fused to a substantial portion of the minimized domain of tetanus toxin fragment C, unless the context clearly dictates that said term refers to the protein sequence. The term "CEA-DOM fusion protein" refers to a polypeptide encoded by a CEA-DOM fusion as described. The terms "CEA-DOM fusion" and "CEA-DOM fusion protein" are also understood to refer to fragments thereof, homologs thereof, and functional equivalents thereof (collectively referred to as "variants"), such as those in which one or more amino acids is inserted, deleted or replaced by other amino acid(s). The CEA-DOM fusions of the present invention, upon administration to a mammal such as a human being, can stimulate an immune response by helper T cells or cytotoxic T cells, or stimulate the production of antibodies at least as well as a "wild-type" CEA sequence. In preferred embodiments of the invention, the CEA-DOM fusion can enhance the immune response as compared to a wild-type CEA.

The abbreviation "AD" refers to the anchoring domain of a CEA gene or protein. The anchoring domain of the wild-type human CEA is located from about amino acid 679 to about amino acid 702 of SEQ ID NO:30. An amino acid sequence encoding a human CEA devoid of its anchoring domain, herein designated CEAΔAD, is set forth in SEQ ID NO:3.

The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with the molecules of the present invention, including the nucleic acid molecules described herein and the fusion proteins that

are encoded by said nucleic acid molecules. Encompassed by the term "disorder" are chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. The molecules of the present invention are intended for use as treatments for disorders or conditions characterized by aberrant cell proliferation, including, but not limited to, pancreatic cancer, liver cancer, breast cancer, colorectal cancer, ovarian cancer, cervical cancer, and lung cancer.

The term "effective amount" means sufficient vaccine composition is introduced to produce the adequate levels of the polypeptide, so that an immune response results. One skilled in the art recognizes that this level may vary.

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

"hCEA" and "hCEAopt" refer to a human carcinoembryonic antigen and a human codon-optimized carcinoembryonic antigen, respectively.

"Substantially similar" means that a given nucleic acid or amino acid sequence shares at least 75%, preferably 85%, more preferably 90%, and even more preferably 95% identity with a reference sequence. In the present invention, the reference sequence can be relevant portions of the wild-type human CEA nucleotide or amino acid sequence, or the wild-type nucleotide or amino acid sequence of an immunoenhancing element or subunit thereof, such as DOM, as dictated by the context of the text. The reference sequence may also be, for example, the wild-type rhesus monkey CEA sequence. Thus, a CEA protein sequence that is "substantially similar" to the wild-type human CEA protein or fragment thereof will share at least 75% identity with the relevant fragment of the wild-type human CEA, along the length of the fragment, preferably 85% identity, more preferably 90% identity and even more preferably 95% identity. Whether a given CEA, DOM, or other protein or nucleotide sequence is "substantially similar" to a reference sequence can be determined for example, by comparing sequence information using sequence analysis software such as the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482, 1981).

A "substantial portion" of a gene, variant, fragment, or subunit thereof, means a portion of at least 50%, preferably 75%, more preferably 90%, and even more preferably 95% of a reference sequence.

"Immunoenhancing element" refers to a portion of the CEA fusion proteins of the present invention which is capable of stimulating or enhancing the immune response to the associated

CEA protein, relative to full-length wild-type CEA. Immunoenhancing elements of the present invention are selected from the group consisting of: heat shock protein (HSP) 70, lysosome-associated membrane protein (LAMP), fragment C of tetanus toxoid (FrC), the N-terminal domain of FrC (DOM), the heavy fragment of constant chain of immune globulin G1 (FcIgG), the vesicular stomatitis virus glycoprotein (VSV-G), cholera toxin (CT) from *Vibrio cholerae*, and heat labile enterotoxin of *E.coli* (LT). The term "immunoenhancing element" is used interchangeably herein with the term "adjuvant."

A "gene" refers to a nucleic acid molecule whose nucleotide sequence codes for a polypeptide molecule. Genes may be uninterrupted sequences of nucleotides or they may include such intervening segments as introns, promoter regions, splicing sites and repetitive sequences. A gene can be either RNA or DNA. A preferred gene is one that encodes the invention peptide.

The term "nucleic acid" or "nucleic acid molecule" is intended for ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), probes, oligonucleotides, fragment or portions thereof, and primers. DNA can be either complementary DNA (cDNA) or genomic DNA, e.g. a gene encoding a CEA fusion protein.

"Wild-type CEA" or "wild-type protein" or "wt protein" refers to a protein comprising a naturally occurring sequence of amino acids or variant thereof. The amino acid sequence of wild-type human CEA is shown in FIGURE 7E (SEQ ID NO:30). The amino acid sequence of the wild-type rhesus monkey CEA was previously described (WO 2004/072287, SEQ ID NOs: 33 and 34, FIGURES 7C-7D).

"Wild-type CEA gene" refers to a gene comprising a sequence of nucleotides that encodes a naturally occurring CEA protein, including proteins of human origin or proteins obtained from another organism, including, but not limited to, other mammals such as rat, mouse and rhesus monkey. The nucleotide sequence of the human CEA gene is available in the art (*supra*). *See also* Beauchemin et al., *Mol. Cell. Biol.* 7:3221-3230 (1987); Zimmerman et al., *Proc. Natl. Acad. Sci. USA* 84:920-924 (1987); and Thompson et al. *Proc. Natl. Acad. Sci. USA* 84(9):2965-69 (1987). The nucleotide sequence of the wild-type rhesus monkey gene is shown in FIGURES 7A-7B.

The term "mammalian" refers to any mammal, including a human being.

The abbreviation "Ag" refers to an antigen.

The abbreviations "Ab" and "mAb" refer to an antibody and a monoclonal antibody, respectively.

The abbreviation "ORF" refers to the open reading frame of a gene.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Panel A shows a schematic representation of the CEA fusion proteins used in this study. Vectors expressing the CEA fusion proteins were derived from plasmid pV1Jns as described in EXAMPLE 1. The constructs comprise a CEA nucleotide sequence from nt 1 to nt 2037 with a net deletion of 64 aa corresponding to the GPI anchoring sequence and express CEA from aa 1 to

aa 679. The sequence coordinates of each protein fused to CEA are also indicated. Panel B shows expression of pV1J-derived constructs in transfected cells. HeLa cells were transfected with plasmids pV1J/CEA-VSV-G, pV1J/CEA-FcIgG, pV1J/CEA-DOM, pV1J/CEA-HSP70, pV1J/CEA-LAMP, or pV1J/CEA and processed for Western blot analysis as described in EXAMPLE 4. The specificity of the antibody used for Western blot is indicated. The CEA protein is indicated (black arrow). The positions of molecular size standards (in kilodaltons) are also shown.

FIGURE 2 shows a comparison of expression efficiency of the CEA fusion constructs. HeLa cells were transfected with the indicated plasmids and CEA derived protein present in cell lysates (A) and supernatants (B) was measured by ELISA as described in EXAMPLE 4. Results obtained are representative of two independent experiments.

FIGURE 3 shows a comparison of immunogenicity of different constructs encoding CEA fusion proteins. C57BL/6 mice were electroporated intramuscularly with a 5 or 50 μg/dose of the indicated plasmids. Injections were carried out at days 0 and 14. Panel A The number of IFNγ-secreting T cells in PBMC in each individual mouse was determined using a pool of peptides covering aa 497-703 (pool D) as described in EXAMPLES 5 and 10. Average number of IFNγ-secreting T cells are also shown (filled circles). SFC values of the pV1J/CEA-DOM, and pV1J/CEA-FcIgG are significantly different from those of pV1J/CEA. Panel B. Antibody titer was measured by ELISA using purified CEA as substrate. Average values of each cohort immunized with 50 μg dose of the indicated plasmid are shown. Titers that are significantly different from those of mice injected with pV1J/CEA are indicated with an asterisk.

FIGURE 4 shows the induction of CEA-specific immune responses in CEA transgenic mice. Groups of 12 CEA transgenic mice were immunized with plasmid DNA (50 μg/dose electroinjected in the quadriceps muscle) or Adenovirus vectors (10^9 vp/dose) carrying the codon usage optimized cDNA of CEA, CEA-DOM or CEA-FcIgG. CEA-specific $CD8^+$ T cells elicited by the DNA/DNA (A) and Ad/Ad (C) immunization regimen were measured by intracellular IFNγ staining on PBMC of each immunized mouse. The average values for each cohort are also shown (filled circle). The CEA-DOM and CEA-FcIgG cohorts immunized with DNA/DNA and Ad/Ad regimens were significantly different from the CEA vaccinated group. CEA-specific antibody titers of each individual mouse vaccinated with the DNA/DNA (B) or Ad/Ad (D) immunization regimen were measured by ELISA. Titers elicited by CEA-DOM and CEA-FcIgG vectors were significantly different from those elicited by CEA.

FIGURE 5 shows the immunogenicity of the DNA/Ad regimen. Groups of 12 CEA transgenic mice were immunized with plasmid DNA (50 μg/dose) and Adenovirus vectors (10^9 vp/dose) carrying the codon usage optimized cDNA of CEA, CEA-DOM or CEA-FcIgG. CEA-specific $CD8^+$ T cells were measured by intracellular IFNγ staining on PBMC of each immunized mouse (A). The average values for each cohort are also shown (filled circle). The CEA-DOM and CEA-FcIgG cohorts

were significantly different from the CEA vaccinated group. CEA-specific antibody titers of each individual mouse were measured by ELISA (B). Titers elicited by CEA-DOM and CEA-FcIgG vectors were significantly different from those elicited by CEA. Average values are shown (filled circles).

FIGURE 6 shows the detection of $CD4^+$ T cell response to tetanus toxoid protein. CEA transgenic mice were immunized with pV1J/CEA-DOMopt as described in EXAMPLE 11. IFNγ intracellular staining on pooled PBMC from immunized mice was performed with peptide p30. Whole lymphocyte gating and gating for $CD8^+$ (R3) and $CD4^+$ T cells (R4) are shown.

FIGURE 7 shows the antitumor effect of vaccination with vectors carrying codon optimized cDNA of CEA, CEA-DOM or CEA-FcIgG. Groups of 10 CEA transgenic mice were immunized were with DNA/DNA (A), Ad/Ad (B) and DNA/Ad (C) vaccination regimens using plasmid DNA and Ad vectors carrying the codon usage optimized cDNAs of CEA, CEA-DOM or CEA-FcIgG, as described in EXAMPLE 13. Two weeks after the last injection, mice were challenged with sc inoculation of $5x10^5$ MC38-CEA tumor cells. Percentage of tumor free mice in the vaccinated groups was determined at weekly intervals and compared to that of untreated controls. Mice vaccinated with CEA-DOM vectors (DNA/Ad modality) was significantly different from control mice (log rank test $p<0.05$).

FIGURE 8 shows the effect of CD4, CD8, or NK depletion on the induction of anti-tumor effect induced by CEA-DOM DNA/Ad immunization. CEA transgenic mice were immunized with repeated weekly injections of 50 μg of pV1J/CEA-DOMopt followed by a boost with $1x10^9$ vp of Ad-CEA-DOMopt (EXAMPLE 14). One week after the last injection, mice were either not depleted, or were depleted of $CD4^+$ T cells, $CD8^+$ T cells, or NK cells. Two weeks after the last immunization, mice were challenged with sc inoculation of $5x10^5$ MC38-CEA tumor cells. Percentage of tumor free mice in the vaccinated groups was determined at weekly intervals and compared to that of untreated controls. The data indicate that the percentage of tumor-free mice in the vaccinated group was significantly different from untreated controls and depleted cohorts.

FIGURE 9, shows the nucleotide sequence (SEQ ID NO:1) of an exemplary, fully optimized hCEA-DOM fusion. The amino acid sequence of the encoded protein is also shown (SEQ ID NO:25). The CEA portion of the nucleotide sequence of this particular CEA fusion consists of nucleotides 1 to 2037, which are codon-optimized for high-level expression in a human host cell. The DOM portion of the nucleotide sequence is shown in bold and is also codon-optimized for high-level expression in human cells. Junction sequences, created by the cloning strategy employed to fuse the CEA and LTB sequences are underlined.

FIGURE 10 shows an exemplary nucleotide (SEQ ID NO:5) sequence of a hCEA-FcIgGopt fusion. The sequence of the encoded protein (SEQ ID NO:26) is also shown. The CEA portion of the nucleotide sequence of this particular CEA fusion consists of nucleotides 1 to 2037, which are codon-optimized for high-level expression in a human host cell. The FcIgG portion of the nucleotide

sequence, which is also codon-optimized for high-level expression in human cells, is shown in bold. Junction sequences, created by the cloning strategy employed to fuse the CEA and LTB sequences are underlined.

FIGURE 11 shows the nucleotide sequence of a portion of the wild-type human CEA cDNA from nt 1 to nt 2037 (SEQ ID NO:2, Panel A), encoding a portion of the hCEA protein from aa 1 to aa 679 (SEQ ID NO:3, Panel B).

FIGURE 12 shows the non-optimized nucleotide sequence of the minimized domain of tetanus toxin fragment C (DOM) cDNA from nt 1 to nt 825 (SEQ ID NO:27), encoding the DOM protein, also shown (SEQ ID NO:28).

FIGURE 13 shows the non-optimized nucleotide sequence of an exemplary hCEA-DOM fusion (SEQ ID NO:29). The CEA portion of the nucleotide sequence of this particular CEA fusion consists of nucleotides 1 to 2037. The DOM portion of the nucleotide sequence is shown in bold.

FIGURE 14 shows the shows nucleotide sequences of wild-type genes encoding rhesus monkey CEA (Panels A and B, SEQ ID NOs:31 and 32) and the amino acid sequences of the corresponding proteins (Panels C and D, SEQ ID NOs:33 and 34), as previously described (WO 2004/072287). Panel E shows the amino acid sequence of wild-type human CEA (SEQ ID NO:30), which was previously described (*see, e.g.,* U.S. Patent No. 5,274,087).

FIGURE 15 shows an exemplary nucleotide sequence (SEQ ID NO:35) of a rhesus monkey CEA- DOM fusion, herein designated rhCEA-DOMopt. The sequence of the encoded fusion protein (SEQ ID NO:36) is also shown. The CEA portion of the nucleotide sequence of this particular CEA fusion consists of nucleotides 1 to 2037, which are codon-optimized for high-level expression in a human host cell. The DOM portion of the nucleotide sequence, which is also codon-optimized for high-level expression in human cells, is shown in bold.

FIGURE 16 shows an exemplary nucleotide sequence (SEQ ID NO:37) of a rhesus monkey CEA- CTB fusion, herein designated rhCEA-CTBopt. The sequence of the encoded fusion protein (SEQ ID NO:38) is also shown. The CEA portion of the nucleotide sequence of this particular CEA fusion consists of nucleotides 1 to 2037, which are codon-optimized for high-level expression in a human host cell. The CTB portion of the nucleotide sequence, which is also codon-optimized for high-level expression in human cells, is shown in bold.

DETAILED DESCRIPTION OF THE INVENTION

Carcinoembryonic antigen (CEA) is commonly associated with the development of adenocarcinomas. The present invention relates to compositions and methods to elicit or enhance immunity to the protein product expressed by the CEA tumor-associated antigen, wherein aberrant CEA expression is associated with the carcinoma or its development. Association of aberrant CEA expression with a carcinoma does not require that the CEA protein be expressed in tumor tissue at all timepoints of

its development, as abnormal CEA expression may be present at tumor initiation and not be detectable late into tumor progression or vice-versa.

To this end, the present invention provides polynucleotides, vectors, host cells, and encoded proteins comprising a CEA sequence or variant thereof for use in vaccines and pharmaceutical compositions for the treatment and/or prevention of a cancer. The polynucleotides of the present invention comprise a nucleotide sequence encoding a CEA protein or variant thereof, fused to a nucleotide sequence encoding at least a subunit of an immunoenhancing element, such as a bacterial enterotoxin or substantial portion thereof, which can effectively adjuvant an immune response to the associated CEA.

The CEA nucleotide sequences of the present invention can be of human origin or can be a CEA homolog from another species. The wild-type human CEA nucleotide sequence has been reported (*see, e.g.,* U.S. Patent No. 5,274,087; U.S. Patent No 5,571,710; and U.S. Patent No 5,843,761). The rhesus monkey CEA sequence was recently described (WO 2004/072287). The CEA portion of the CEA fusion may be full-length, or any variant sufficient to elicit a CEA-specific immune response in a mammal. CEA variants of the present invention include, but are not limited to, sequences that are C- or N-terminally truncated, sequences with conservative substitutions, and sequences with internal deletions or insertions.

In preferred embodiments of the present invention, the CEA portion of the CEA fusion is human CEA (SEQ ID NO:30) or a functional equivalent thereof, for example, a human CEA deleted of its anchoring domain (SEQ ID NO:3). In other preferred embodiments, the CEA portion is a rhesus monkey CEA (SEQ ID NOs:33 and 34), or functional equivalent thereof.

Accordingly, the present invention relates to a synthetic polynucleotide comprising a sequence of nucleotides encoding a CEA fusion protein, said fusion protein comprising a CEA protein or a biologically active fragment or mutant form of a CEA protein fused to an immunoenhancing element or substantial portion thereof, which can effectively enhance the immune response to the CEA protein. Said mutant forms of the CEA protein include, but are not limited to: conservative amino acid substitutions, amino-terminal truncations, carboxy-terminal truncations, deletions, or additions. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the immunological properties of the CEA protein as set forth in SEQ ID NO:30. The synthetic polynucleotides of the present invention encode mRNA molecules that express a functional CEA fusion protein so as to be useful in the development of a therapeutic or prophylactic cancer vaccine.

In preferred embodiments, the CEA portion of the encoded CEA fusion protein is deleted of its C-terminal anchoring domain (AD) , which is located from about amino acid 679 to about amino acid 702 of the human full-length CEA (SEQ ID NO:30) herein designated CEAΔAD, as set forth in SEQ ID NO:3 and shown in FIGURE 11. While not being bound by theory, deletion of the anchoring domain

increases secretion of the CEA fusion protein, thereby enhancing cross priming of the CEA-LTB immune response.

The immunoenhancing element portion of the CEA fusion proteins of the present invention are capable of stimulating or enhancing the immune response to the associated CEA protein and are selected from the group consisting of: heat shock protein (HSP) 70, lysosome-associated membrane protein (LAMP), fragment C of tetanus toxoid (FrC), the N-terminal domain of FrC (DOM), the heavy fragment of constant chain of immune globulin G1 (FcIgG), the vesicular stomatitis virus glycoprotein (VSV-G), cholera toxin (CT) from *Vibrio cholerae*, and heat labile enterotoxin of *E.coli* (LT). In preferred embodiments of the present invention, the adjuvant portion of the CEA fusion comprises the N-terminal domain of FrC (DOM), which has been shown to strongly potentiate the immunogenicity of codelivered antigens. In further preferred embodiments, the adjuvant portion of the CEA fusion is a substantial portion of FcIgG.

A CEA fusion comprising a truncated human CEA fused to a single epitope of tetanus toxin (Q830 – L844) has been described (Lund et al. *Cancer Gene Therapy* 10: 365-376 (2003)). Unlike this single-epitope fusion, the CEA fusions of the present invention comprise a substantial portion of an immunoenhancing element or subunit thereof, as described above, which is capable of enhancing the immunogenicity of a CEA protein or variant thereof. A substantial portion of an immunoenhancing element to be used for the compositions and methods described herein does not include portions that are less than 50% of a full-length immunoenhancing element or subunit thereof. The strategy used herein, which utilizes full-length adjuvant subunits or substantial portions thereof, was employed to ensure a greater immune response to the fused CEA sequence. While not being bound by theory, it is believed that if the immunoenhancing element chosen as adjuvant comprises greater than one helper epitope, limiting the adjuvant sequence of the fusion protein to a single epitope would arguably lead to a reduced effect on the immunogenicity of the target protein. Additionally, it is believed that if the adjuvant-mediated enhancement of the immune response is dependent on the interaction of the immunoenhancing element with specific cell receptors and not based on a universal epitope, then the receptor interaction could depend on a specific structural configuration that would require a substantial portion of the immunoenhancing element to exert an adjuvant effect. In such a case, a short adjuvant sequence comprising a single epitope would be insufficient in mediating an increase of the immune response.

Also contemplated for use in the present invention are nucleotide sequences encoding variants or mutants of the immunoenhancing elements described herein, including, but not necessarily limited to: nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations. In some cases, it may be advantageous to add specific point mutations to the nucleotide sequence encoding the adjuvant or subunit to reduce or eliminate toxicity of the encoded protein.

The immunoenhancing element, subunit, or substantial portion thereof may be fused to the amino terminus or the carboxy terminus of the CEA sequence. Further, the immunoenhancing element sequence and the CEA sequence can be fused N-terminus to N-terminus, C-terminus to C-terminus, C-terminus to N-terminus or N-terminus to N-terminus. In preferred embodiments of the present invention, the C-terminus of the CEA polypeptide is fused to the N-terminus of the immunoenhancing element.

The present invention relates to a synthetic nucleic acid molecule (polynucleotide) comprising a sequence of nucleotides which encodes mRNA that expresses a novel CEA fusion protein; for example, nucleotide sequences encoding the fusion proteins as set forth in SEQ ID NOs:25, 26, 36 and 38. The nucleic acid molecules of the present invention are substantially free from other nucleic acids.

The present invention also relates to recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification. The synthetic DNA molecules, associated vectors, and hosts of the present invention are useful for the development of a cancer vaccine.

Exemplary nucleic acid molecules of the present invention comprise a nucleotide sequence selected from the group consisting of: SEQ ID NOs: 1, 5, 29, 35, and 37, as shown in FIGUREs 9, 10, 13, 15, and 16, which encode exemplary CEA fusion proteins of the present invention.

The present invention also includes biologically active fragments or mutants of SEQ ID NOs: 1, 5, 29, 35, and 37, which encode mRNA expressing exemplary CEA fusion proteins. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the pharmacological properties of the hCEA protein, including but not limited to the hCEA protein as set forth in SEQ ID NO:30. Any such polynucleotide includes but is not necessarily limited to: nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations. The mutations of the present invention encode mRNA molecules that express a functional CEA fusion protein in a eukaryotic cell so as to be useful in cancer vaccine development.

Also included within the scope of this invention are mutations in the DNA sequence that do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in the functionality of the polypeptide.

As stated above, the present invention further relates to recombinant vectors that comprise the nucleic acid molecules disclosed throughout this specification. These vectors may be comprised of DNA or RNA. For most cloning purposes, DNA vectors are preferred. Typical vectors include plasmids, modified viruses, baculovirus, bacteriophage, cosmids, yeast artificial chromosomes, and other forms of episomal or integrated DNA that can encode a CEA fusion protein. It is well within

the purview of the skilled artisan to determine an appropriate vector for a particular gene transfer or other use.

Also provided by the present invention are purified CEA fusion proteins encoded by the nucleic acids disclosed throughout this specification. In exemplary embodiments of this aspect of the invention, the CEA fusion protein comprises a sequence of amino acids selected from the group consisting of: SEQ ID NOs: 25, 26, 36 and 38.

Included in the present invention are DNA sequences that hybridize to the complement of SEQ ID NOs: 1, 5, 29, 35, and 37 under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows. Prehybridization of filters containing DNA is carried out for about 2 hours to overnight at about 65°C in buffer composed of 6x SSC, 5x Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for about 12 to 48 hrs at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 x 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for about 1 hour in a solution containing 2x SSC, 0.1% SDS. This is followed by a wash in 0.1x SSC, 0.1% SDS at 50°C for 45 minutes before autoradiography. Other procedures using conditions of high stringency would include either a hybridization step carried out in 5x SSC, 5x Denhardt's solution, 50% formamide at about 42°C for about 12 to 48 hours or a washing step carried out in 0.2x SSPE, 0.2% SDS at about 65°C for about 30 to 60 minutes. Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd Edition; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, (1989) or Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Plainview, NY (2001). In addition to the foregoing, other conditions of high stringency which may be used are also well known in the art.

An expression vector containing a CEA fusion protein-encoding nucleic acid molecule may be used for high-level expression of CEA fusion protein in a recombinant host cell. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Also, a variety of bacterial expression vectors may be used to express recombinant CEA fusion sequences in bacterial cells if desired. In addition, a variety of fungal cell expression vectors may be used to express recombinant CEA fusion sequences in fungal cells. Further, a variety of insect cell expression vectors may be used to express recombinant protein in insect cells.

The present invention also relates to host cells transformed or transfected with vectors comprising the nucleic acid molecules of the present invention. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of bovine, porcine, monkey and rodent origin; and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Such

recombinant host cells can be cultured under suitable conditions to produce a CEA fusion protein or a biologically equivalent form. In a preferred embodiment of the present invention, the host cell is human. As defined herein, the term "host cell" is not intended to include a host cell in the body of a transgenic human being, human fetus, or human embryos.

As noted above, an expression vector containing DNA encoding a CEA fusion protein may be used for expression of CEA fusion protein in a recombinant host cell. Therefore, another aspect of this invention is a process for expressing a CEA fusion protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid comprising a sequence of nucleotides that encodes a CEA fusion protein into a suitable human host cell, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element or subunit thereof, wherein the immunoenhancing element or subunit thereof is selected from the group consisting of: heat shock protein (HSP) 70, lysosome-associated membrane protein (LAMP), fragment C of tetanus toxoid (FrC), the N-terminal domain of FrC (DOM), the heavy fragment of constant chain of immune globulin G1 (FcIgG), the vesicular stomatitis virus glycoprotein (VSV-G), cholera toxin (CT) from *Vibrio cholerae*, and heat labile enterotoxin of *E.coli* (LT); and wherein the fusion protein is capable of producing an immune response in a mammal; and, (b) culturing the host cell under conditions which allow expression of said CEA fusion protein.

A preferred immunoenhancing element for use in this aspect of the invention is DOM. Another preferred immunoenhancing element is FcIgG.

In a further preferred embodiment of this aspect of the invention, the nucleotide sequence of the CEA portion of the fusion and/or the immunoenhancing element portion of the fusion are codon-optimized for high-level expression in human cells.

This invention also provides a process for expressing a CEA-DOM fusion protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid comprising a sequence of nucleotides that encodes a CEA-DOM fusion protein into a suitable human host cell, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of the N-terminal domain of fragment C of tetanus toxin (DOM), and wherein the fusion protein is capable of producing an immune response in a mammal; and, (b) culturing the host cell under conditions which allow expression of said CEA-DOM fusion protein.

In preferred embodiments of the process for expressing a CEA-DOM fusion protein described above, the DOM portion is codon-optimized for high-level expression in human cells. In other preferred embodiments, the CEA portion of the CEA fusion is codon-optimized for high-level expression in human cells. In still further preferred embodiments, both the CEA and the DOM portions are codon-optimized for high-level expression in human cells.

Following expression of a CEA fusion in a host cell, CEA fusion protein may be recovered to provide CEA fusion protein in active form. Several protein purification procedures are

available and suitable for use. Recombinant protein may be purified from cell lysates and extracts by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography. In addition, recombinant CEA fusion protein can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for a CEA protein, or polypeptide fragments of a CEA protein.

The nucleic acid molecules comprising CEA fusions and the encoded fusion proteins of this invention were designed to enhance the CEA-specific immune response, relative to full-length CEA cDNA, for use in vaccine development. To further enhance the immunogenic properties of the CEA fusion sequences of the present invention, in some embodiments described herein, the polynucleotides encoding CEA fusion proteins comprise optimized codons for further high level expression in a host cell, as described below. In these embodiments, at least a portion of the codons of the CEA fusions are designed so as to use the codons preferred by the projected host cell, which in preferred embodiments is a human cell. The optimized CEA fusions may be used for the development of recombinant adenovirus or plasmid-based DNA vaccines, which provide effective immunoprophylaxis against CEA-associated cancer through neutralizing antibody and cell-mediated immunity. The synthetic molecules may be used as an immunogenic composition. This invention provides codon-optimized CEA fusion polynucleotides which, when directly introduced into a vertebrate *in vivo*, including mammals such as primates and humans, induce the expression of encoded proteins within the animal.

As stated above, in some embodiments of the present invention, the synthetic molecules comprise a sequence of nucleotides, wherein some of the nucleotides have been altered so as to use the codons preferred by a human cell, thus allowing for high-level fusion protein expression in a human host cell. The synthetic molecules may be used as a source of a CEA fusion protein, for example, CEA-DOM fusion protein, which may be used in a cancer vaccine to provide effective immunoprophylaxis against CEA-associated carcinomas through neutralizing antibody and cell-mediated immunity. The nucleic acid molecules disclosed herein may also serve as the basis for a DNA-based cancer vaccine.

A "triplet" codon of four possible nucleotide bases can exist in over 60 variant forms. Because these codons provide the message for only 20 different amino acids (as well as transcription initiation and termination), some amino acids can be coded for by more than one codon, a phenomenon known as codon redundancy. For reasons not completely understood, alternative codons are not uniformly present in the endogenous DNA of differing types of cells. Indeed, there appears to exist a variable natural hierarchy or "preference" for certain codons in certain types of cells. As one example, the amino acid leucine is specified by any of six DNA codons including CTA, CTC, CTG, CTT, TTA, and TTG. Exhaustive analysis of genome codon frequencies for microorganisms has revealed endogenous DNA of *E. coli* most commonly contains the CTG leucine-specifying codon, while the DNA of yeasts and slime molds most commonly includes a TTA leucine-specifying codon. In view of this

hierarchy, it is generally believed that the likelihood of obtaining high levels of expression of a leucine-rich polypeptide by an *E. coli* host will depend to some extent on the frequency of codon use. For example, it is likely that a gene rich in TTA codons will be poorly expressed in *E. coli*, whereas a CTG rich gene will probably be highly expressed in this host. Similarly, a preferred codon for expression of a leucine-rich polypeptide in yeast host cells would be TTA.

The implications of codon preference phenomena on recombinant DNA techniques are manifest, and the phenomenon may serve to explain many prior failures to achieve high expression levels of exogenous genes in successfully transformed host organisms--a less "preferred" codon may be repeatedly present in the inserted gene and the host cell machinery for expression may not operate as efficiently. This phenomenon suggests that synthetic genes which have been designed to include a projected host cell's preferred codons provide an optimal form of foreign genetic material for practice of recombinant DNA techniques. Thus, one aspect of this invention is a CEA fusion gene that is codon-optimized for expression in a human cell. In a preferred embodiment of this invention, it has been found that the use of alternative codons encoding the same protein sequence may remove the constraints on expression of exogenous CEA fusion protein in human cells.

In accordance with some embodiments of the present invention, the nucleic acid molecules which encode the CEA fusion proteins are converted to a polynucleotide sequence having an identical translated sequence but with alternative codon usage as described by Lathe, "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data: Theoretical and Practical Considerations" *J. Molec. Biol.* 183:1-12 (1985), which is hereby incorporated by reference. The methodology generally consists of identifying codons in the wild-type sequence that are not commonly associated with highly expressed human genes and replacing them with optimal codons for high expression in human cells. The new gene sequence is then inspected for undesired sequences generated by these codon replacements (e.g., "ATTTA" sequences, inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, etc.). Undesirable sequences are eliminated by substitution of the existing codons with different codons coding for the same amino acid. The synthetic gene segments are then tested for improved expression.

The methods described above were used to create synthetic gene sequences which encode CEA fusion proteins of the present invention, resulting in a gene comprising codons optimized for high level expression. While the above procedure provides a summary of our methodology for designing codon-optimized genes for use in cancer vaccines, it is understood by one skilled in the art that similar vaccine efficacy or increased expression of genes may be achieved by minor variations in the procedure or by minor variations in the sequence.

One of skill in the art will also recognize that additional nucleic acid molecules may be constructed that provide for high levels of CEA fusion expression in human cells, wherein only a portion of the codons of the DNA molecules are codon-optimized. For example, in some embodiments of the

present invention, codons comprising the CEA portion of the CEA fusion are optimized for high-level expression in human cells, and codons comprising the adjuvant portion of the CEA fusion are substantially similar to the wild-type adjuvant-encoding nucleotide sequence . In other embodiments of the present invention, codons comprising the adjuvant portion of the CEA fusion are optimized for high-level expression in human cells, and codons comprising the CEA portion of the CEA fusion are substantially similar to a wild-type CEA gene. In still other embodiments of the present invention, both the CEA and the adjuvant portions of the CEA fusion are codon-optimized for high-level expression in human cells. CEA fusions in which only a subset of codons are optimized within the CEA and/or the adjuvant portion of the CEA fusion are also contemplated by this invention.

The nucleic acids of the present invention may be assembled into an expression cassette which comprises sequences designed to provide for efficient expression of the protein in a human cell. The cassette preferably contains CEA fusion protein-encoding gene, with related transcriptional and translations control sequences operatively linked to it, such as a promoter, and termination sequences. In a preferred embodiment, the promoter is the cytomegalovirus promoter without the intron A sequence (CMV), although those skilled in the art will recognize that any of a number of other known promoters such as the strong immunoglobulin, or other eukaryotic gene promoters may be used. A preferred transcriptional terminator is the bovine growth hormone terminator, although other known transcriptional terminators may also be used. The combination of CMV-BGH terminator is particularly preferred.

In accordance with this invention, the CEA fusion expression cassette is inserted into a vector. The vector is preferably an adenoviral or plasmid vector, although linear DNA linked to a promoter, or other vectors, such as adeno-associated virus or a modified vaccinia virus, retroviral or lentiviral vector may also be used.

If the vector chosen is an adenovirus, it is preferred that the vector be a so-called first-generation adenoviral vector. These adenoviral vectors are characterized by having a non-functional E1 gene region, and preferably a deleted adenoviral E1 gene region. In some embodiments, the expression cassette is inserted in the position where the adenoviral E1 gene is normally located. In addition, these vectors optionally have a non-functional or deleted E3 region. It is preferred that the adenovirus genome used be deleted of both the E1 and E3 regions (ΔE1ΔE3). The adenoviruses can be multiplied in known cell lines which express the viral E1 gene, such as 293 cells, or PERC.6 cells, or in cell lines derived from 293 or PERC.6 cell which are transiently or stablily transformed to express an extra protein. For examples, when using constructs that have a controlled gene expression, such as a tetracycline regulatable promoter system, the cell line may express components involved in the regulatory system. One example of such a cell line is T-Rex-293; others are known in the art.

For convenience in manipulating the adenoviral vector, the adenovirus may be in a shuttle plasmid form. This invention is also directed to a shuttle plasmid vector which comprises a plasmid portion and an adenovirus portion, the adenovirus portion comprising an adenoviral genome

which has a deleted E1 and optional E3 deletion, and has an inserted expression cassette comprising a CEA fusion protein encoding nucleotide sequence. In preferred embodiments, there is a restriction site flanking the adenoviral portion of the plasmid so that the adenoviral vector can easily be removed. The shuttle plasmid may be replicated in prokaryotic cells or eukaryotic cells.

In a preferred embodiment of the invention, the expression cassette is inserted into the pMRKAd5-HV0 adenovirus plasmid (*See* Emini et al., WO 02/22080, which is hereby incorporated by reference). This plasmid comprises an Ad5 adenoviral genome deleted of the E1 and E3 regions. The design of the pMRKAd5-HV0 plasmid was improved over prior adenovectors by extending the 5' cis-acting packaging region further into the E1 gene to incorporate elements found to be important in optimizing viral packaging, resulting in enhanced virus amplification. Advantageously, this enhanced adenoviral vector is capable of maintaining genetic stability following high passage propagation.

Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the adenoviruses, shuttle plasmids, and DNA immunogens of this invention.

It has been determined in accordance with the present invention that the CEA fusion protein-encoding molecules described herein (e.g. SEQ ID NO:1) are expressed with equivalent efficiency compared to the corresponding wild type CEA sequence (*See* EXAMPLE 4). It has also been shown herein that plasmids pV1J/hCEA-DOM and pV1J/hCEA-FcIgG elicited a greater CEA-specific cell-mediated and humoral immune response than CEA (*See* EXAMPLE 10). It has also been shown in accordance with the present invention that tolerance to the CEA self antigen can be broken more efficiently with the Dom and FcIgG CEA fusions described herein, relative to the full-length wild-type CEA cDNA, due to the increased immunogenic properties of the CEA fusions. The enhanced immunogenic properties of these fusion proteins was observed upon immunization with DNA or Ad vectors, indicating that enhanced immunogenicity of CEA-LT fusions is not limited to plasmid DNA immunization (*see* EXAMPLE 11).

Therefore, the vectors described above may be used in immunogenic compositions and vaccines for preventing the development of adenocarcinomas associated with aberrant CEA expression and/or for treating existing cancers. The vectors of the present invention allow for vaccine development and commercialization by eliminating difficulties with obtaining high expression levels of exogenous CEA in successfully transformed host organisms and by providing a CEA fusion protein which can elicit an enhanced immune response when administered to a mammal such as a human being.

To this end, one aspect of the instant invention is a method of preventing or treating CEA-associated cancer comprising administering to a mammal a vaccine vector comprising a polynucleotide comprising a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element selected from the group consisting of: heat shock protein (HSP) 70, lysosome-associated membrane protein (LAMP), fragment C of tetanus toxoid (FrC), the N-terminal

domain of FrC (DOM), the heavy fragment of constant chain of immune globulin G1 (FcIgG), the vesicular stomatitis virus glycoprotein (VSV-G), cholera toxin (CT) from *Vibrio cholerae*, and heat labile enterotoxin of *E.coli* (LT); and wherein the fusion protein is capable of producing an immune response in a mammal.

In preferred embodiments of the methods described herein, the Immunoenhancing element is DOM or FcIgG.

In accordance with the method described above, the vaccine vector may be administered for the treatment or prevention of a cancer in any mammal, including but not limited to: lung cancer, breast cancer, and colorectal cancer. In a preferred embodiment of the invention, the mammal is a human.

Further, one of skill in the art may choose any type of vector for use in the treatment and prevention method described. Preferably, the vector is an adenovirus vector or a plasmid vector. In a preferred embodiment of the invention, the vector is an adenoviral vector comprising an adenoviral genome with a deletion in the adenovirus E1 region, and an insert in the adenovirus E1 region, wherein the insert comprises an expression cassette comprising: (a) a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element or substantial portion thereof; wherein the immunoenhancing element is selected from the group consisting of: heat shock protein (HSP) 70, lysosome-associated membrane protein (LAMP), fragment C of tetanus toxoid (FrC), the N-terminal domain of FrC (DOM), the heavy fragment of constant chain of immune globulin G1 (FcIgG), the vesicular stomatitis virus glycoprotein (VSV-G), cholera toxin (CT) from *Vibrio cholerae*, and heat labile enterotoxin of *E.coli* (LT); and wherein the fusion protein is capable of producing an immune response in a mammal; and (b) a promoter operably linked to the polynucleotide.

The instant invention further relates to an adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising: (a) a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element or substantial portion thereof; wherein the immunoenhancing element is selected from the group consisting of: HSP70, LAMP, FrC, DOM, the FcIgG, VSV-G, CT and LT; and wherein the fusion protein is capable of producing an immune response in a mammal; and (b) a promoter operably linked to the polynucleotide.

In a preferred embodiment of this aspect of the invention, the adenovirus vector is an Ad 5 vector.

In another preferred embodiment of the invention, the adenovirus vector is an Ad 6 vector.

In yet another preferred embodiment, the adenovirus vector is an Ad 24 vector.

Also contemplated for use in the present invention is an adenovirus vaccine vector comprising a adenovirus genome that naturally infects a species other than human, including, but not limited to, chimpanzee adenoviral vectors. A preferred embodiment of this aspect of the invention is a chimp Ad 3 vaccine vector.

In another aspect, the invention relates to a vaccine plasmid comprising a plasmid portion and an expression cassette portion, the expression cassette portion comprising: (a) a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element or substantial portion thereof, selected from the group consisting of: HSP70, LAMP, FrC, DOM, the FcIgG, VSV-G, CT and LT; and wherein the fusion protein is capable of producing an immune response in a mammal; and (b) a promoter operably linked to the polynucleotide.

In some embodiments of this invention, the recombinant adenovirus and plasmid-based polynucleotide vaccines disclosed herein are used in various prime/boost combinations in order to induce an enhanced immune response. In this case, the two vectors are administered in a "prime and boost" regimen. For example the first type of vector is administered one or more times, then after a predetermined amount of time, for example, 2 weeks, 1 month, 2 months, six months, or other appropriate interval, a second type of vector is administered one or more times. Preferably the vectors carry expression cassettes encoding the same polynucleotide or combination of polynucleotides. In the embodiment where a plasmid DNA is also used, it is preferred that the vector contain one or more promoters recognized by mammalian or insect cells. In a preferred embodiment, the plasmid would contain a strong promoter such as, but not limited to, the CMV promoter. The synthetic CEA fusion gene or other gene to be expressed would be linked to such a promoter. An example of such a plasmid would be the mammalian expression plasmid V1Jns as described (J. Shiver *et. al.* in *DNA Vaccines*, M. Liu et al. eds., N.Y. Acad. Sci., N.Y., 772:198-208 (1996), which is herein incorporated by reference).

As stated above, an adenoviral vector vaccine and a plasmid vaccine may be administered to a vertebrate as part of a single therapeutic regime to induce an immune response. To this end, the present invention relates to a method of protecting a mammal from a CEA-associated cancer comprising: (a) introducing into the mammal a first vector comprising: i) a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element selected from the group consisting of: HSP70, LAMP, FrC, DOM, the FcIgG, VSV-G, CT and LT; and wherein the fusion protein is capable of producing an immune response in a mammal; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element selected from the group consisting of: HSP70, LAMP, FrC,

DOM, the FcIgG, VSV-G, CT and LT; and wherein the fusion protein is capable of producing an immune response in a mammal; and ii) a promoter operably linked to the polynucleotide.

In one embodiment of the method of protection described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid.

In the method described above, the first type of vector may be administered more than once, with each administration of the vector separated by a predetermined amount of time. Such a series of administration of the first type of vector may be followed by administration of a second type of vector one or more times, after a predetermined amount of time has passed. Similar to treatment with the first type of vector, the second type of vector may also be given one time or more than once, following predetermined intervals of time.

The instant invention further relates to a method of treating a mammal suffering from a CEA-associated adenocarcinoma comprising: (a) introducing into the mammal a first vector comprising: i) a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element selected from the group consisting of: HSP70, LAMP, FrC, DOM, the FcIgG, VSV-G, CT and LT; and wherein the fusion protein is capable of producing an immune response in a mammal; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element selected from the group consisting of: HSP70, LAMP, FrC, DOM, the FcIgG, VSV-G, CT and LT; and wherein the fusion protein is capable of producing an immune response in a mammal; and ii) a promoter operably linked to the polynucleotide.

In one embodiment of the method of treatment described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid.

In preferred embodiments of the methods described above, the vectors comprise a sequence of nucleotides that encode a CEA-DOM fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of a DOM subunit. In further preferred embodiments, the vector comprises a sequence of nucleotides that encodes a CEA-FcIgG fusion protein.

The amount of expressible DNA or transcribed RNA to be introduced into a vaccine recipient will depend partially on the strength of the promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10 μg to 300 μg of a plasmid vaccine vector is administered directly into

muscle tissue. An effective dose for recombinant adenovirus is approximately $10^6 - 10^{12}$ particles and preferably about 10^7—10^{11}particles. Subcutaneous injection, intradermal introduction, impression though the skin, and other modes of administration such as intraperitoneal, intravenous, intramuscular or inhalation delivery are also contemplated.

In preferred embodiments of the present invention, the vaccine vectors are introduced to the recipient through intramuscular injection.

It may be desirable for the vaccine vectors of the present invention to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, it may be advantageous to administer an agent which assists in the cellular uptake of DNA, such as, but not limited to calcium ion. These agents are generally referred to as transfection facilitating reagents and pharmaceutically acceptable carriers. Those of skill in the art will be able to determine the particular reagent or pharmaceutically acceptable carrier as well as the appropriate time and mode of administration.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

EXAMPLE 1

Plasmid constructs.

pV1J/CEA$_{opt}$ and pV1J/CEA: These two constructs carry the codon usage optimized and wild type cDNA of CEA, respectively. The CEA coding sequence is located between the CMV/intronA immediate early promoter of cytomegalovirus and the bovine growth hormone polyadenylation signal. All the constructs encoding CEA fusion proteins were generated by fusing the CEA cDNA from nt 1 to nt 2037 (SEQ ID NO:2), corresponding to aa 1 to aa 679 (SEQ ID NO:3), with the cDNA fragment corresponding to the following: fragment C of tetanus toxoid (CEA-FrC, SEQ ID NO:4), the N-terminal domain of FrC (CEA-DOM, SEQ ID NO:1), the heavy fragment of constant chain of immune globulin G1 (CEA-FcIgG, SEQ ID NO:5), the lysosome-associated membrane protein (CEA-LAMP, SEQ ID NO:6), the heat shock protein 70 (CEA-HSP70, SEQ ID NO:7), or the vesicular stomatitis virus glycoprotein (CEA-VSV-G, SEQ ID NO:8).

pV1J/CEA-FrC, pV1J/CEA-DOM, pV1J/CEA-FcIgG, pV1J/CEA-LAMP, pV1J/CEA-HSP70 and pV1J/CEA-VSV-G: FrC and DOM coding sequences were obtained by PCR amplification from pRep-TeT.C plasmid as described in Rice et al. (*J. Immunol.* 169: 3908-13 (2002)). FcIgG was obtained from total RNA of human PBMC. VSV-G and HSP70 were obtained from p-FAST-VSV-G and from plasmid pY3111, respectively. LAMP1 was obtained by gene assembly. Amplifications were carried out using the following primers: FrC sense (5'-T A T *T C T A G A* T T C A A C A C C A A T T C C A T T T T C T T A T T C -3' (SEQ ID NO:9) FrC antisense (5'-G C G G C C G C T A G A A T C A T T T G T C C A T C C T T C A T C -3' (SEQ ID NO:10), DOM sense (5'-T A T *T C T A G A* T T C A A C A C C A A T T C C A T T T T C T T A T T C -3' (SEQ ID NO:11) DOM antisense (5- T T A G C G G C C G C T A G T T C T G T A T C A T A T C G T A A A G G G -3' (SEQ ID NO:12), FcIgG sense (5'-*T C T A G A* T A A A A C T C A C A C A T G C C C A -3' (SEQ ID NO:13) FcIgG antisense (5'-G C C G A C T C A T T T A C C C G G A G A C A G G G A G -3' (SEQ ID NO:14), LAMP sense (5-*T C T A G A* T T T G A T C C C C A T T G C T G T G G G C G G T G C C C T G -3' (SEQ ID NO:15) LAMP antisense (5'- G G C G T G A C T C C T C T T C C T G C C A A T G A G G T A G G C A A T G A G -3' (SEQ ID NO:16), VSV-G sense (5'-A T A *T C T A G A* T T T C A C C A T A G T T T T T C C A C A C A A C C -3' (SEQ ID NO:17) VSV-G antisense (5'-G C G G C C G C C T T C C T T C C A A G T C G G T T C A T C T C T A T G -3' (SEQ ID NO:18), HSP70 sense (5'-G C *T C T A G A* T A T G G C T C G T G C G G T C G G G A T C G A C C -3' (SEQ ID NO:19)) and HSP70 antisense (5'G C C G C G G C C G C T C A C T T G G C C T C C C G G C C G T C G T C G -3' (SEQ ID NO:20). The amplified DNA was introduced at the 3' end of the CEA coding sequence generating plasmids pV1J/CEA-FrC, pV1J/CEA-DOM, pV1J/CEA-FcIgG, pV1J/CEA-LAMP, pV1J/CEA-HSP70 and pV1J/CEA-VSV-G.

pV1J/CEA-DOMopt and pV1J/CEA-FcIgGopt: The codon usage optimized cDNA of DOM and FcIgG were synthesized by oligonucleotide assembly (Geneart GmbH, Regensburg, Germany) and cloned in pCR-script vector (Stratagene, La Jolla, CA). To generate pV1J/CEA-DOMopt, DOM_{opt} was amplified by PCR using the following primers: Dom_{opt} sense (5'- G T T A *T C T A G A* A G C A C C C C A T C C C -3' (SEQ ID NO:21)) and Dom_{opt} reverse (5'- T T A A G A T C T C T A A G A T C T G G T G T C G T A T C T C A G G G G -3' (SEQ ID NO:22). The amplified product was then inserted into the *XbaI/BglII* sites of plasmid pV1J/CEAopt. To generate pV1J/CEA-FcIgGopt, $FcIgG_{opt}$ was amplified by PCR using the following primers: $FcIgG_{opt}$ sense (5'-T T A *T C T A G A* A A G A C C C A C A C C T G C C C C C C T T G C -3' (SEQ ID NO:23))and as $FcIgG_{opt}$ reverse (5'- T A T A G A T C T T A G G G T A C C T T A C T T G C C G G G G -3' (SEQ ID NO:24)) the amplified product was inserted into *XbaI/BglII* sites of plasmid pV1J/CEAopt.

EXAMPLE 2

Adenovirus Vectors.

Ad5/CEA$_{opt}$: Plasmid pCR-CEAopt was digested with *Eco*RI. The resulting 2156 bp insert was purified and cloned into the *Eco*RI of the polyMRK-Ad5 shuttle plasmid.

Ad5/CEA: The shuttle plasmid pMRK-CEA for generation of Ad5 vector was obtained by digesting plasmid pDelta1sp1B/CEA with *Ssp*I and *Eco*RV. The 9.52 kb fragment was then ligated to a 1272 bp *Bgl*II/*Bam*HI-restricted, Klenow-treated product from plasmid polyMRK. A *Pac*I/*Stu*I fragment from pMRK-CEA and pMRK-CEAopt containing the expression cassette for CEA and E1 flanking Ad5 regions was recombined to *Cla*I linearized plasmid pAd5 in BJ5183 *E. coli* cells. The resulting plasmids were pAd5-CEA and pAd5-CEAopt, respectively. Both plasmids were cut with *Pac*I to release the Ad ITRs and transfected in PerC-6 cells. Amplification of Ad5 vectors was carried out by serial passage. MRKAd5/CEA and MRKAd5/CEAopt or fusion were purified through standard CsCl gradient purification and extensively dialyzed against A105 buffer (5mM Tris-Cl pH 8.0, 1mM $MgCl_2$, 75 mM NaCl, 5% Sucrose, 0.005 Tween 20).

Ad5/CEA-DOMopt and Ad5/CEA-FcIgGopt: Plasmid pMRK-CEA-DOMopt and pMRK-CEA-FcIgGopt were constructed by cutting polyMRK-Ad5 shuttle plasmid with *Swa*I and by ligating the linearized vector with the 2.9 kb DNA fragment derived from pV1J/CEA-DOMopt or ligating the linearized vector with the 2700 bp DNA fragment derived from pV1J/CEA-FcIgG1opt that had been restricted with *Eco*RI, *Bgl*II and treated with Klenow. pMRK-CEA-FcIgGopt and pMRK-CEA-DOMopt were linearized and recombined into the Ad genome as indicated above.

EXAMPLE 3

Construction of CEA fusion proteins.

To determine the immunogenicity of CEA fusion proteins, a series of vectors were constructed encoding aminoacids 1 to 679 of the human CEA protein fused to a panel of selected polypeptides (see EXAMPLE 1). These sequences were chosen in view of their reported immunoenhancing properties, which have been demonstrated in a variety of experimental systems. The CEA fusions were engineered by joining the cDNA of the CEA protein deleted of the GPI anchoring sequence to the foreign polypeptides (FIGURE 1A). The tumor antigen was linked to the HSP70, FcIgG or LAMP sequences to determine whether enhancement of antigen uptake or retargeting to the endosomal compartment would lead to an increased immune response. Similarly, fusion to fragment C of tetanus toxin (FrC) or to a minimal domain devoid of potentially competitive MHC class I binding epitopes (DOM) (Rice et al. *J. Immunol.* 169: 3908-13 (2002)) was constructed to promote humoral and $CD4^+$ T cell responses. Lastly, CEA was linked to the VSV-G coding sequence to determine whether fusion to a viral glycoprotein would influence the immunogenic properties of CEA.

The coding sequences of the CEA-fusions were cloned into vectors pV1Jns under the control of the human CMV/intron A promoter plus the bovine growth hormone (BGH) polyadenylation

signal (EXAMPLE 1). Plasmids pV1J/CEA-FRC, pV1J/CEA-DOM, pV1J/CEA-FcIgG, pV1J/CEA-LAMP, pV1J/CEA-VSV-G, and pV1J/CEA-HSP70 carry the wild type cDNA of CEA fused to the coding sequences of the indicated foreign polypeptides.

EXAMPLE 4

Detection of CEA expression.

CEA expression was monitored by ELISA and Western blot analysis. Expression of the fusion proteins in transfected cells was examined by Western blot analysis using antibodies specific for CEA, VSV-G, FcIgG, tetanus toxin, or HSP70. HeLa cells were either transfected with the indicated plasmid or infected with the selected Ad vector. After 48 hr incubation, whole cell lysates and culture supernatant were harvested.

CEA expression in cell lysate or supernatant was also monitored using the Direct Elisa CEA Kit (DBC-Diagnostics Biochem Canada Inc). CEA protein was detected with the antibody specific for the fused polypeptide in transfected cell lysates, whereas no expression of the target antigen was observed in the mock transfected control samples (FIGURE 1B). The molecular mass of the fusion proteins did not differ significantly from that of CEA. This apparent lack of difference in molecular mass between the various CEA polypeptides is probably due to the high degree of glycosylation of the tumor antigen.

To compare the efficiency of expression of the vectors encoding the CEA-fusions to that of pV1J/CEA, HeLa cells were transfected with the different plasmids and CEA expression of these constructs was determined two days post transfection by ELISA. Plasmids pV1J/CEA-FrC, pV1J/CEA-DOM, pV1J/CEA-FcIgG, pV1J/CEA-LAMP, pV1J/CEA-VSV-G and pV1J/CEA-HSP70 expressed CEA with comparable efficiency as pV1J/CEA (FIGURE 2A). Most of the fusion proteins were secreted and detected in the cell supernatant; however, CEA-LAMP was not released from the transfected cells, probably due to its re-routing to the lysosomal compartment. (FIGURE 2B). Thus, these results indicate that the cDNA encoding the CEA-FrC, CEA-DOM, CEA-VSV-G, CEA-FcIgG, CEA-HSP70 and CEA-LAMP fusion proteins are expressed with equivalent efficiency to that of the cDNA encoding the full length CEA protein.

EXAMPLE 5

Peptides.

Lyophilized CEA peptides were purchased from Bio-Synthesis and resuspended in DMSO at 40 mg/ml. Pools of peptides of 15 aa overlapping by 11 residues were assembled as described (Facciabene et al. *J. Virol.* 78: 8663-72 (2004)). Final concentrations were the following: pool A = 1.2 mg/ml, pool B 0.89 mg/ml, pool C 0.89 mg/ml, pool D 0.8 mg/ml. Immune response to DOM was

monitored using the tetanus toxoid peptide p30 (F_{947}NNFTVSFWLRVPKVSASHLE_{967} (SEQ ID NO:25)) (Rice et al. *J. Immunol.* 167: 1558-65 (2001)). Peptides were stored at –80°C.

EXAMPLE 6

Mice immunization and tumor challenge.

All animal studies were approved by the IRBM institutional animal care and use committee. Female C57BL/6 mice (H-2^b) were purchased from Charles River (Lecco, Italy) CEA transgenic mice (H-2^b) were provided by J. Primus (Vanderbilt University) and kept in standard conditions (Clarke et al. *Cancer Res.* 58:1469-77 (1998)).

C57BL/6 mice were subjected to two DNA injections in quadriceps muscle followed by electrical stimulation as previously described (Rizzuto et al. *Proc. Natl. Acad. Sci. USA* 96 (11): 6417-22 (1999)). Injections were carried out at three-week intervals. CEA transgenic mice were subjected to either 5 weekly injections of plasmid DNA (50μg/injection), 2 injections of Ad vectors ($1x10^9$ viral particles/injection), or 5 weekly injections followed by a boost with Ad. Two weeks after the last injection, humoral and cell mediated immune response were analyzed. Mice were also challenged with a subcutaneous (s.c.) injection of $5x10^5$ MC38-CEA cells (Clarke et al., *supra*). At weekly intervals, mice were examined for tumor growth.

EXAMPLE 7

Antibodies detection and titration.

Sera for antibody titration were obtained by retro-orbital bleeding. ELISA assay was performed using highly purified CEA protein as previously described (Facciabene et al., *supra*). Anti-CEA serum titers were calculated as the reciprocal limiting dilution of serum producing an absorbance at least 3-fold greater than the absorbance of autologous pre-immune serum at the same dilution.

EXAMPLE 8

IFN-γ ELISPOT assay.

Assays were carried out using mouse splenocytes and CEA-specific peptides as previously described (Facciabene et al., *supra*). Briefly, purified splenocytes were plated in duplicate in 96-wells MAIP plates (Millipore Corp., Billerica, MA) at a density of $5x10^5$ and $2.5x10^5$ cells/well. Cells were incubated for 20 h at 37°C with 1μg/ml suspension of each peptide. Concanavalin A was used as positive internal control for each mouse at 5μg/ml. Plates were washed and incubated for 12hrs at 4°C with 50μl/well of biotin-conjugated rat anti-mouse IFNγ. After extensive washing, 50 μl/well of NBT/B-CIP (Pierce Biotechnology, Inc., Rockford, IL) were added until development of spots was clearly

visible. Plates were then washed extensively and air dried. Spots were then counted using an automated ELISPOT reader.

EXAMPLE 9

Cytokine intracellular staining.

One to two millions mouse PBMC or splenocytes in 1ml RPMI 10% FCS were incubated with the indicated pool of peptides (5-6 μg/ml final concentration of each peptide) and brefeldin A (1 μg/ml; BD Pharmingen) at 37°C for 12-16 hours as previously described (Facciabene et al., *supra*). Cells were washed, stained with surface antibodies, fixed, permeabilized and incubated with IFNγ-FITC antibodies (BD Pharmingen). Cells were fixed with 1% formaldehyde solution in PBS and analyzed on a FACS-Calibur flow cytometer, using CellQuest software (Becton Dickinson, San Jose, CA).

EXAMPLE 10

CEA-DOM and CEA-FcIgG fusions enhance the immunogenicity of the CEA protein.

To examine the immune responses induced by the plasmids encoding CEA-FRC, CEA-DOM, CEA-VSV-G, CEA-FcIgG, CEA-HSP70 and CEA-LAMP fusions, groups of 9 C57BL/6 mice were immunized with two i.m. injections of 50 or 5 μg of each plasmid. The immunizations were three weeks apart. In view of the enhanced transduction and immunogenicity reported with electroporation (Zucchelli et al. *J. Virology* 74:11598 (2000), Widera et al., *J. Immunol.* 164: 4635 (2000)), plasmid DNAs were routinely electroporated (DNA-EP) into mouse skeletal muscle.

The immune response elicited by different plasmids was measured by IFNγELISPOT assay, 2 weeks after the last injection. Antigen-specific IFNγ secretion from stimulated splenocytes was measured using a pool of 15mer peptides overlapping by 11 aa and encompassing the C-terminal region of CEA (pool D, aa 497-703) (Zucchelli et al., *supra*). The analysis of the immune response to CEA was carried out with peptide pool D since the cellular immune response to CEA in C57BL/6 mice is primarily biased towards the C-terminal region of this protein (Zuccelli et al., *supra*). As a negative control, cytokine production was also measured upon stimulation of the splenocytes with DMSO at the same concentration utilized to solubilize the CEA peptides.

Injection of pV1J/CEA-DOM or pV1J/CEA-Fc elicited a greater immune response to CEA as compared to pV1J/CEA. The greater immunogenicity of these two fusion proteins resulted in higher geometric mean values of spot forming cells (SFC) per 10^6 splenocytes (FIGURE 3A). Plasmids pV1J/CEA-DOM and pV1J/CEA-FcIgG had similar immunogenic properties and exerted a 3- to 4-fold increase in CEA-specific immune responses upon injection of 5 or 50 μg of plasmid DNA (pV1J/CEA-DOM: 590 and 1098 SFC/10^6 splenocytes, pV1J/CEA-FcIgG: 510 and 1160, pV1J/CEA: 146 and 264 SFC/10^6 splenocytes, respectively). No significant differences were noted between the SFC values

elicited by the pV1J/CEA-FrC, pV1J/CEA-LAMP, pV1J/CEA-HSP70 and pV1J/CEA. No CEA specific immune responses were detected in negative control samples.

To determine the effect of the CEA-fusions on the humoral response to CEA, sera from immunized mice were tested in ELISA using purified CEA protein as substrate (FIGURE 3B). An increase in CEA-specific antibody titer was observed upon injection of 50 μg of plasmids pV1J/CEA-DOM, pV1J/CEA-FcIgG, pV1J/CEA-FrC and pV1J/CEA-HSP70. On the contrary, injection of pV1J/CEA-LAMP and pV1J/CEA-VSV-G resulted in a CEA-specific antibody response similar to that observed upon immunization with pV1J/CEA. Taken together, these data demonstrate that fusion of the CEA coding sequence to the DOM or FcIgG cDNA results in an increase in the CEA-specific cell mediated and humoral immune response.

EXAMPLE 11

CEA-DOM and CEA-FcIgG fusions break tolerance to target antigen in CEA transgenic mice.

Tolerance to the target antigen is one of the hurdles that a cancer vaccine must overcome to elicit an immune response and to exert an efficient antitumor effect. Thus, it was deemed appropriate to determine whether the enhanced immunogenic properties of CEA-DOM and CEA-FcIgG fusions would break tolerance to CEA more efficiently than the CEA protein. To this end, CEA transgenic mice were utilized to perform comparative immunization studies. These transgenic mice carry the entire human CEA gene and flanking sequences and express the CEA protein in the intestine, mainly in the cecum and colon. This mouse line is a useful model for studying the safety and efficacy of immunotherapy strategies directed against this tumor self antigen (Clarke et al., *supra*).

In view of the enhanced immunogenic properties of vectors carrying the codon usage optimized cDNA (cDNAopt) of CEA, both plasmid and Adenovirus vectors were engineered to carry the cDNAopt of the CEA-DOM (CEA-DOMopt) or CEA-FcIgG (CEA-FcIgGopt) fusions. As observed for CEA, CEA-DOMopt and CEA-FcIgGopt cDNAs were shown to be expressed with a greater efficiency of the corresponding wild type cDNA leading to an enhanced immune response to CEA (data not shown).

The immunogenicity of these two fusion proteins was compared to that of CEA by a series of immunization studies based on the use of plasmid DNA and Ad vectors administered either alone or in combination. Cohorts of CEA transgenic mice were immunized with the following varying regimens: i) 5 injections at weekly intervals of 50 μg of plasmid DNA (DNA/DNA), ii) 2 biweekly injections of Adenovirus in doses ranging from $1x10^7$ to $1x10^9$ viral particles (vp) of Adenovirus (Ad/Ad), or iii) 5 weekly injections of plasmid DNA followed by a final injection of $1x10^9$ vp of Adenovirus (DNA/Ad). Immune responses were analyzed by intracellular IFNγstaining on PBMC or splenocytes of each immunized mouse using pool D peptides. Additionally, the induction of CEA-specific antibodies was monitored by ELISA.

DNA/DNA immunization of the CEA transgenic mice revealed that the CEA-DOMopt and CEA-FcIgGopt vectors exerted a measurable $CD8^+$ T cell response to the target antigen (FIGURE 4A). Thus, both constructs were able to break tolerance to CEA in these mice. The antigen specific response elicited by CEA-DOM and CEA-FcIgG fusion proteins was comparable as indicated by the average values of IFNγ intracellular staining (0.22 and 0.34%, respectively). Nonetheless, the immune response elicited by these two constructs was greater than that observed upon vaccination with pV1J/CEAopt (0.07%). Similarly, anti-CEA humoral response was also greater upon vaccination with the fusion proteins. CEA-specific antibody titer was detected in all mice immunized with pV1J/CEA-DOMopt and pV1J/CEA-FcIgGopt and the average of the antibody titer was 56,136 and 24,725, respectively. By contrast, the pV1J/CEAopt immunized group showed an at least a 77 fold lower average titer of CEA-specific antibody (318) (FIGURE 4B).

CEA transgenic mice treated with the Ad/Ad vaccination regimen also showed a better efficiency in breaking tolerance to CEA upon vaccination with CEA-DOMopt and CEA-FcIgGopt Ad vectors than with Ad-CEAopt. A CEA-specific $CD8^+$ T cell response could be observed in the vaccinated mice upon injection of a little as 10^7 vp of Ad-CEA-DOM or Ad-CEA-FcIgG, the CEA-specific response was comparable between the two antigens, and increased upon injection of 10^9 vp (1.55% and 1.15%, respectively). By contrast, 10^9 vp of Ad-CEAopt were necessary to elicit significant $CD8^+$ T-cell precursor frequencies (2.1%) (FIGURE 4C). CEA-specific antibodies were detected in all mice immunized with Ad-CEA-DOMopt and Ad-CEA-FcIgGopt. The averages of the antibody titer were 19,600 and 33,000, respectively. Injection of Ad-CEAopt resulted in a measurable CEA-specific response in only 2 of the treated mice, and the antibody titer was significantly lower (Zucchelli et al., *supra*) (FIGURE 4D). Interestingly, the DNA/Ad immunization showed reduced differences in the $CD8^+$ T cell precursor frequencies elicited by CEA, CEA-DOM and CEA-FcIgG vectors (FIGURE 5A). However, averages of CEA-specific antibody titers were greater upon vaccination with vectors expressing CEA-DOM and CEA-FcIgG than CEA (31,200, 26,120 and 412, respectively) (FIGURE 5B).

Interestingly, regardless of the antigen, no obvious $CD4^+$ cell Th1 response to CEA was detected in any of the three vaccination regimens (data not shown). However, significant $CD4^+$ cell Th1 response against to the helper epitope, p30, present within DOM sequences (Rice et al., *J. Immunol.* 167: 1558-65 (2001)) were detected after DNA/DNA vaccination (0.4%) (FIGURE 6).

Thus, these data demonstrate that the CEA-DOM and CEA-FcIgG fusion proteins can break tolerance to CEA in transgenic mice with greater efficacy than the CEA protein. The enhanced immunogenic properties of these fusion proteins can be observed upon immunization with DNA or Ad vectors. However, the greater ability of these two fusion proteins in eliciting $CD8^+$ T cells to CEA can be overcome, at least in part, by DNA/Ad vaccination regimen.

EXAMPLE 12

T-cell depletion studies.

Immunized animals were depleted of $CD4^+$ T cells, $CD8^+$ T cells, NK cells, by i.p. injection of anti-CD4 (GK1.5 hybridoma), anti-CD8 (Lyt 2.2 hybridoma), or anti Asialo GM1 (Wako Chemicals, Richmond, VA) as described (Perricone et al., *J. Immunother.* 27(4):273-81 (2004); Yoon et al., *J. Ethnopharmacol.* 93 (2-3):247-53 (2004)). Antibodies (100 µl diluted ascitic fluid/dose) were injected on day -7 relative to the tumor challenge and then injected every week for 3 weeks after injection of $5x10^5$ MC38-CEA cells. Depletion conditions were validated by flow cytometry analysis of peripheral blood using phycoerythrin-conjugated MAbs anti-CD4, anti-CD8, and anti-NK (PharMingen, San Diego, CA); 99% of the relevant cell subset was depleted, whereas all other subsets remained within normal levels.

EXAMPLE 13

CEA-DOM immunization exerts an antitumor effect in CEA transgenic mice.

We next ascertained whether the increased immunogenicity of the CEA-DOM or CEA-FcIgG fusions would also lead to an enhanced therapeutic effect, capable of interfering with tumor progression. For this purpose, groups of 10 CEA transgenic mice were subjected to the DNA/DNA, Ad/Ad or DNA/Ad immunization regimens with the CEA-DOM, CEA-FcIgG, or CEA vectors. Two weeks after the last immunization, the CEA transgenic mice were challenged with a subcutaneous injection of $5x10^5$ MC38-CEA cells, a syngenic tumor cell line that expresses CEA (Clarke et al., *supra*). Immunization with DNA/DNA or Ad/Ad modalities did not result in any significant antitumor effect, regardless of the protein expressed by the injected vectors (FIGURE 7). In contrast, DNA-EP/Ad immunization with vectors encoding the CEA-DOM fusion protein resulted in a significant antitumor effect with 7 out of 10 treated mice remaining tumor free by day 34 post challenge. Thus, these results indicate that the enhanced CEA-specific immune response associated with the CEA-DOMopt cDNA, and the DNA/Ad vaccination regimen correlate with a significant antitumor effect in CEA transgenic mice.

EXAMPLE 14

The CEA-DOM antitumor effect is dependent on $CD4^+$ T cells, $CD8^+$ T cells and NK cells.

The effector cells involved in the antitumor effect observed upon DNA-EP and Ad immunization with vectors encoding CEA-DOM fusion were characterized. After DNA/Ad immunization, but prior to tumor challenge, mice were depleted of $CD4^+$, $CD8^+$ T cells, or NK cells by MAbs. Antibodies were given during the course of tumor challenge to ensure continued depletion of the relevant NK and T cell subsets. The depletion of all three cell types was monitored by flow cytometry analysis using antibodies specific for cell surface markers (data not shown). Depletion of $CD4^+$, $CD8^+$ T cells, or NK cells had a negative effect on survival of the immunized mice resulting in the drastic reduction of tumor-free mice as compared to the vaccinated group (FIGURE 8). Thus, these data

indicate that NK, $CD4^+$ and $CD8^+$ T cells play an important role in the antitumor effect exerted by CEA-DOM vaccination.

EXAMPLE 15

Statistical analysis.

Where indicated, results were analyzed by the log rank or two tailed Student t test. A p value < 0.05 was considered significant.

WHAT IS CLAIMED IS:

1. A nucleic acid molecule comprising a sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a substantial portion of an immunoenhancing element selected from the group consisting of: DOM, FcIgG, CT, and LT; and wherein the fusion protein is capable of producing an immune response in a mammal.

2. The nucleic acid molecule of claim 1, wherein the CEA protein is a human CEA protein or variant thereof.

3. The nucleic acid molecule of claim 1, wherein the CEA protein is a rhesus monkey CEA protein or variant thereof.

4. The nucleic acid molecule of claim 1, wherein the CEA protein is C-terminally truncated.

5. The nucleic acid molecule of claim 4, wherein the C-terminal truncation comprises amino acids 679 – 702 of SEQ ID NO:3.

6. The nucleic acid molecule of claim 2, wherein the immunoenhancing element comprises a substantial portion of DOM.

7. The nucleic acid molecule of claim 2, wherein the immunoenhancing element comprises a substantial portion of FcIgG.

8. The nucleic acid molecule of claim 1, wherein the sequence of nucleotides is selected from the group consisting of SEQ ID NOs:1, 5, 29, 35, and 37.

9. A nucleic acid molecule that hybridizes under high stringency conditions to the complement of the nucleic acid molecule of claim 8.

10. A vector comprising the nucleic acid molecule of claim 1.

11. The vector of claim 10, wherein the vector is an adenovirus vector or a plasmid vector.

12. The vector of claim 11, wherein the vector is an Ad 5 vector.

13. The vector of claim 11, wherein the vector is an Ad 6 vector.

14. The vector of claim 11, wherein the vector is pV1JnsB.

15. A host cell comprising the vector of claim 11.

16. A process for expressing a CEA fusion protein in a recombinant host cell, comprising:

(a) introducing a vector comprising the nucleic acid molecule of claim 1 into a suitable host cell; and,

(b) culturing the host cell under conditions which allow expression of said CEA fusion protein.

17. A purified CEA fusion protein encoded by the nucleic acid molecule of claim 1.

18. The purified CEA fusion protein of claim 17, wherein the fusion protein comprises a sequence of amino acids selected from the group consisting of: SEQ ID NOS:25, 26, 36, and 38.

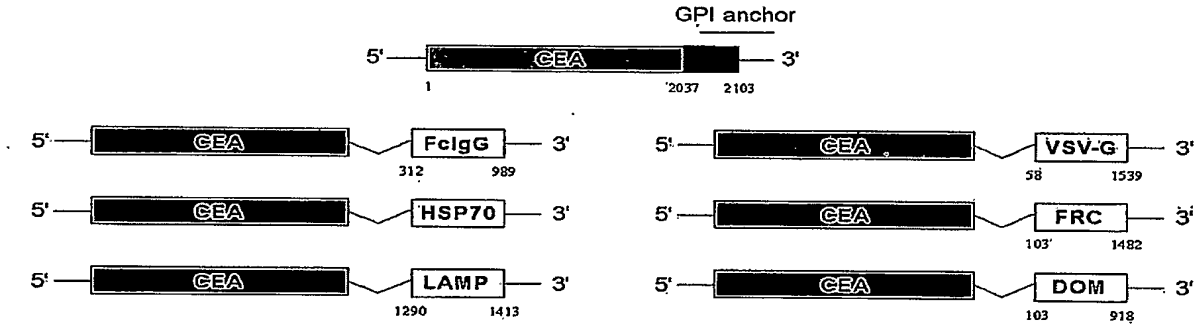
19. The use of the nucleic acid molecule of claim 1 in a mediciament for the treatment of cancer.

20. A method of preventing or treating cancer comprising administering to a mammal the nucleic acid molecule of claim 1.

ABSTRACT OF THE DISCLOSURE

Polynucleotides encoding carcinoembryonic antigen (CEA) fusion proteins are provided, the CEA fusion proteins comprising a CEA protein, or functional variant thereof, fused to a substantial portion of an immunoenhancing element as described herein. The polynucleotides of the present invention can elicit an immune response in a mammal, which, in preferred embodiments, is stronger than the immune response elicited by a wild-type CEA. The gene encoding CEA is commonly associated with the development of human carcinomas. The present invention provides compositions and methods to elicit or enhance immunity to the protein product expressed by the CEA tumor-associated antigen, wherein aberrant CEA expression is associated with a carcinoma or its development. This invention specifically provides adenoviral vector and plasmid constructs carrying polynucleotides encoding CEA fusion proteins and discloses their use in vaccines and pharmaceutical compositions for preventing and treating cancer.

A



B

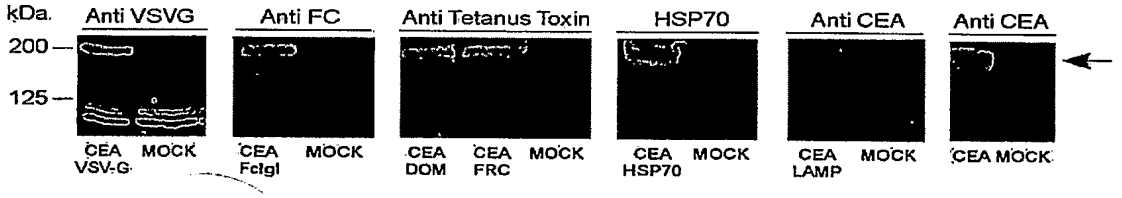


FIGURE 1

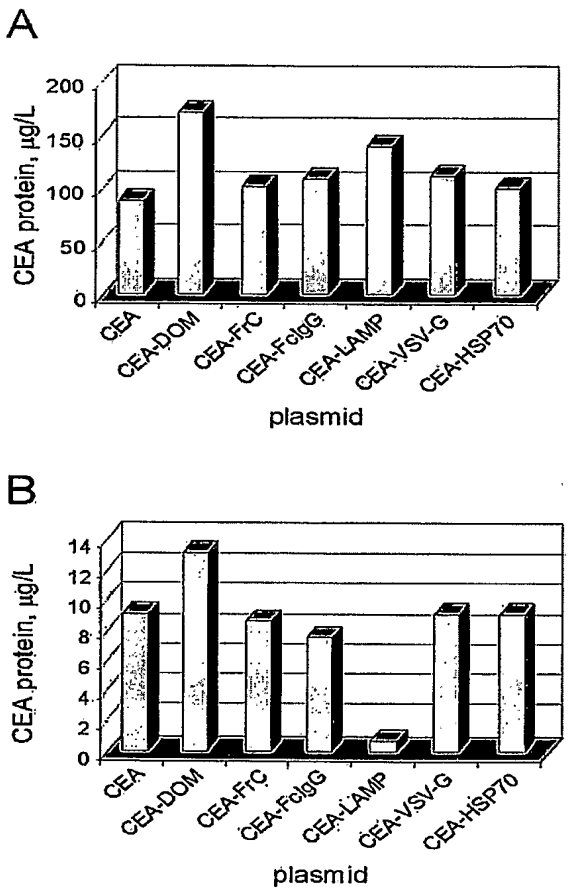


FIGURE 2

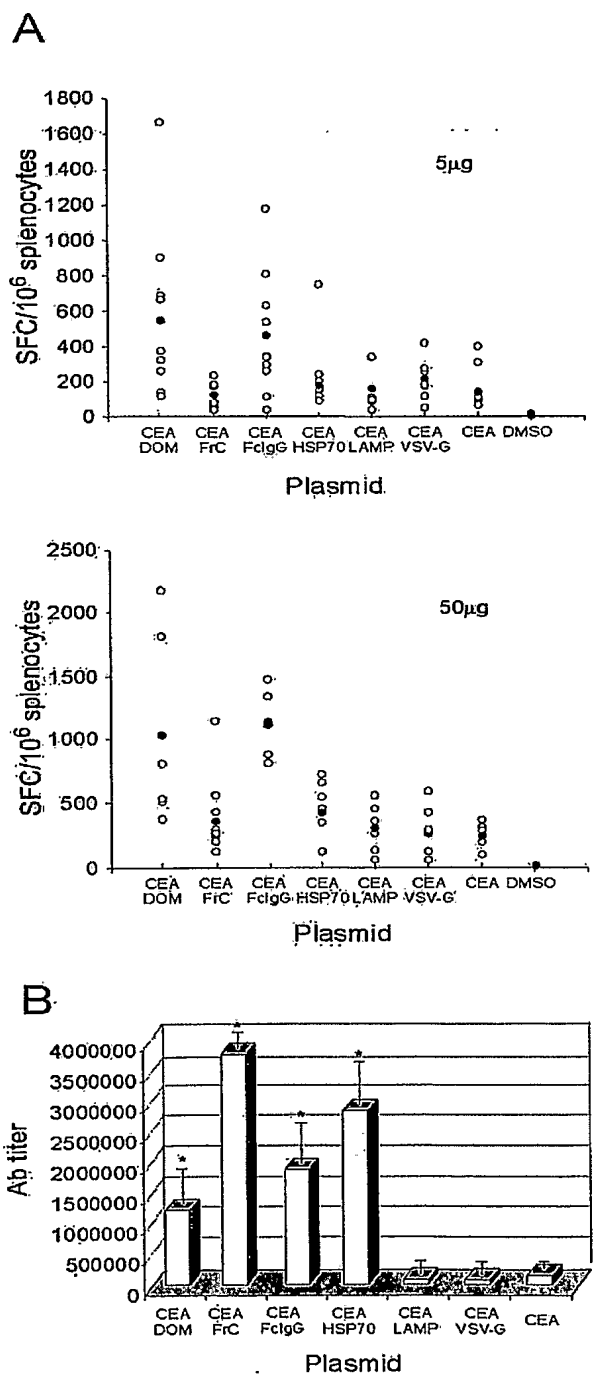


FIGURE 3

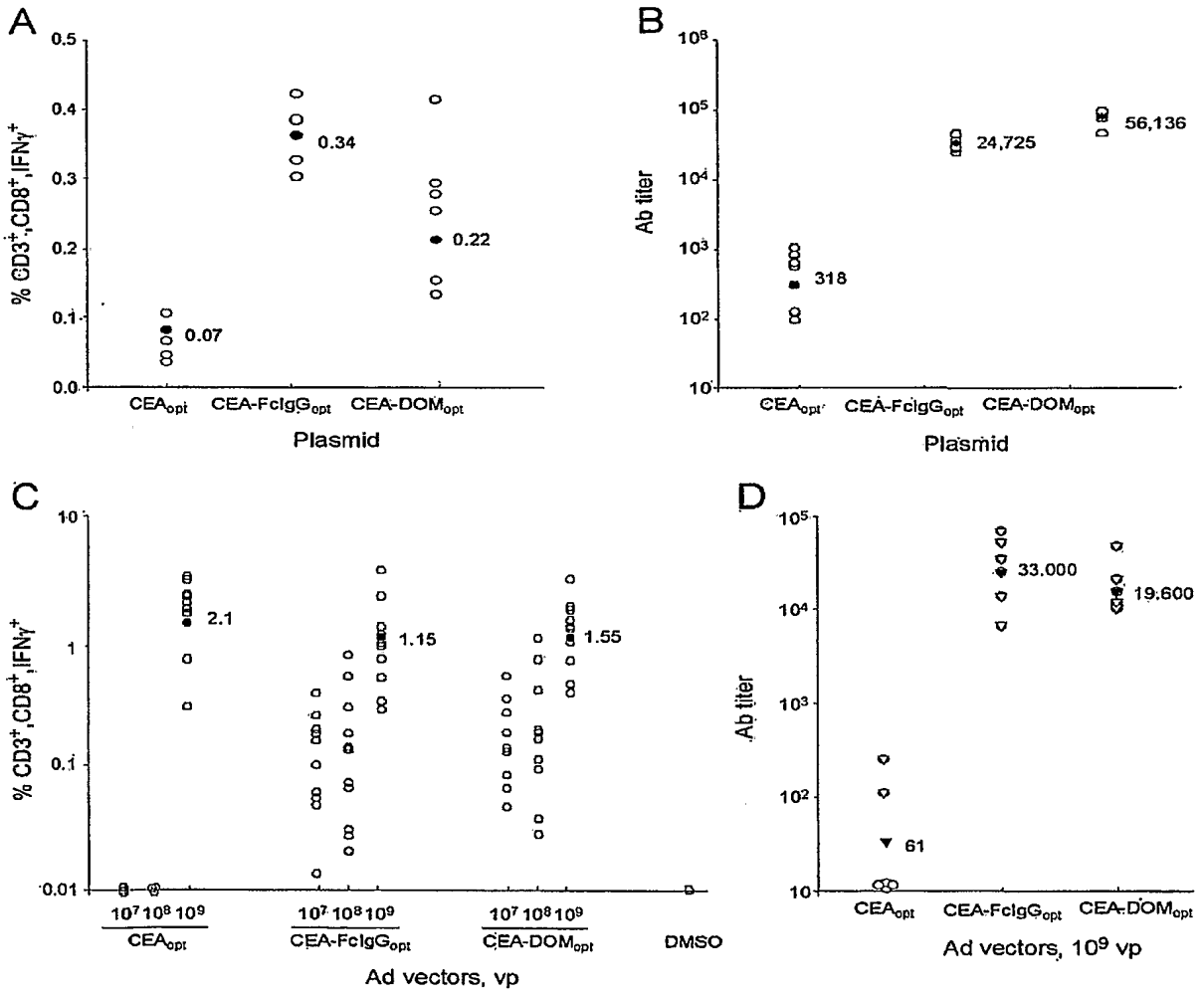


FIGURE 4

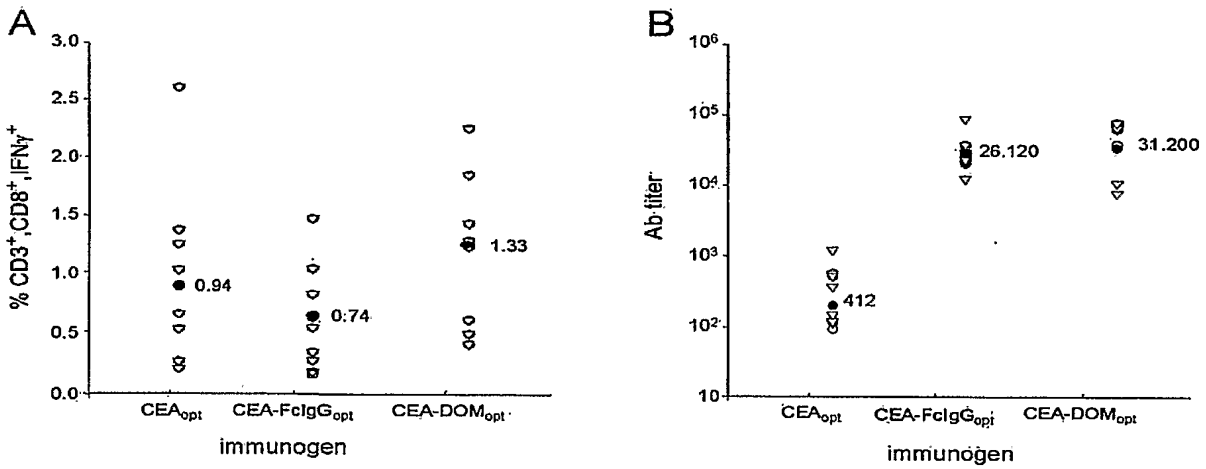


FIGURE 5

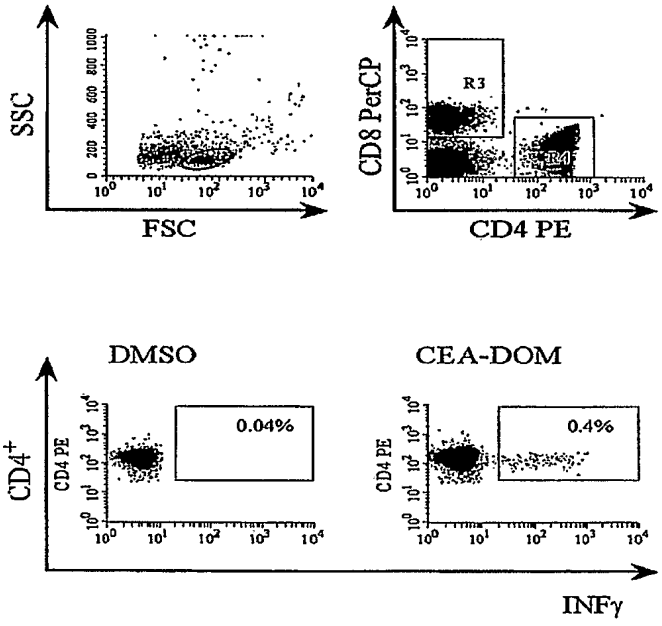


FIGURE 6

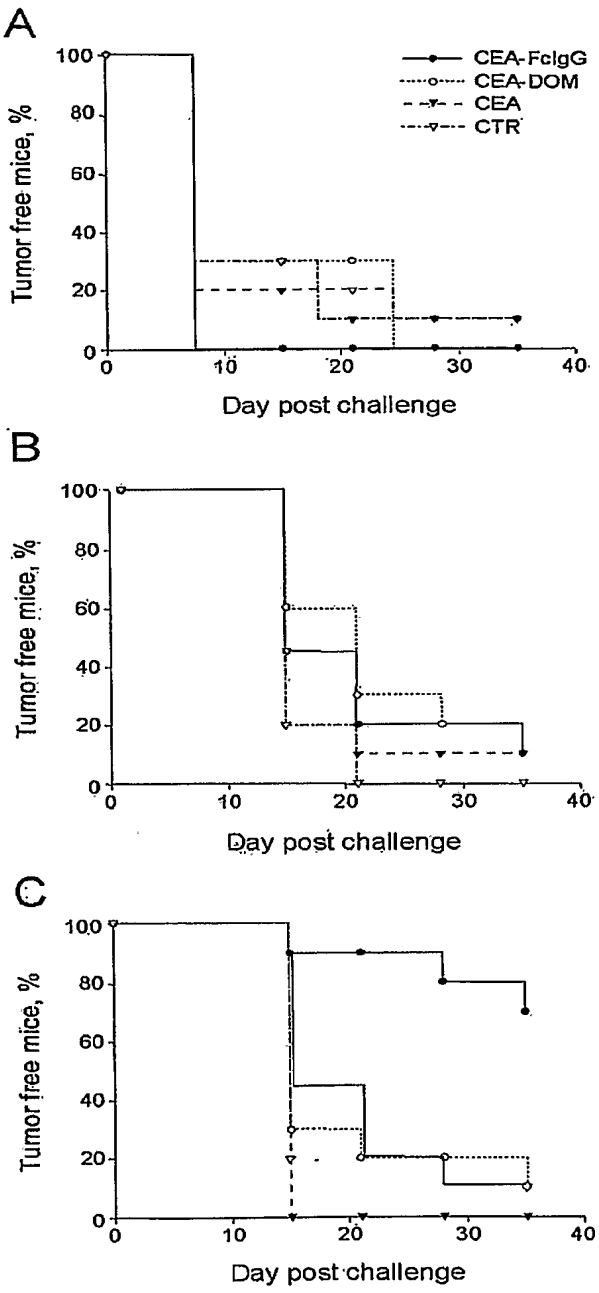


FIGURE 7

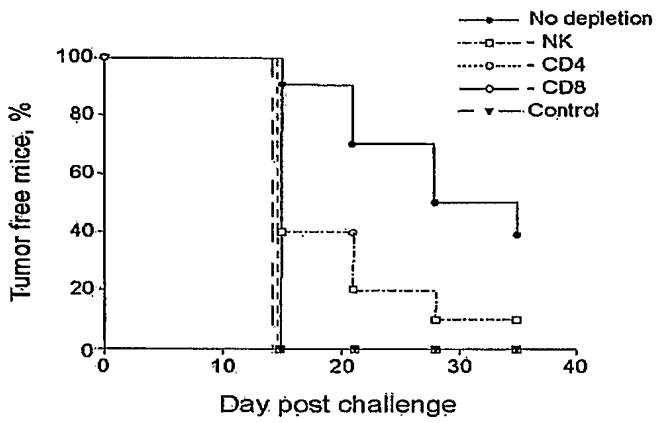


FIGURE 8

```
          M  E  S  P   S  A  P   P  H  R   W  C  I  P   W  Q  R ·
1         ATGGAGAGCC CCAGCGCCCC CCCCCACCGC TGGTGCATCC CCTGGCAGCG
          · L  L  L   T  A  S  L   L  T  F   W  N  P   P  T  T  A ·
51        CCTGCTGCTG ACCGCCAGCC TGCTGACCTT CTGGAACCCC CCCACCACCG
          · K  L  T   I  E  S   T  P  F  N   V  A  E   G  K  E
101       CCAAGCTGAC CATCGAGAGC ACCCCCTTCA ACGTGGCCGA GGGCAAGGAG
           V  L  L  L   V  H  N   L  P  Q   H  L  F  G   Y  S  W ·
151       GTGCTGCTGC TGGTGCACAA CCTGCCCCAG CACCTGTTCG GCTACAGCTG
          · Y  K  G   E  R  V  D   G  N  R   Q  I  I   G  Y  V  I ·
201       GTACAAGGGC GAGCGCGTGG ACGGCAACCG CCAGATCATC GGCTACGTGA
          · G  T  Q   Q  A  T   P  G  P  A   Y  S  G   R  E  I
251       TCGGCACCCA GCAGGCCACC CCCGGCCCCG CCTACAGCGG CCGCGAGATC
           I  Y  P  N   A  S  L   L  I  Q   N  I  I  Q   N  D  T ·
301       ATCTACCCCA ACGCCAGCCT GCTGATCCAG AACATCATCC AGAACGACAC
          · G  F  Y   T  L  H  V   I  K  S   D  L  V   N  E  E  A ·
351       CGGCTTCTAC ACCCTGCACG TGATCAAGAG CGACCTGGTG AACGAGGAGG
          · T  G  Q   F  R  V   Y  P  E  L   P  K  P   S  I  S
401       CCACCGGCCA GTTCCGCGTG TACCCCGAGC TGCCCAAGCC CAGCATCAGC
           S  N  N  S   K  P  V   E  D  K   D  A  V  A   F  T  C ·
451       AGCAACAACA GCAAGCCCGT GGAGGACAAG GACGCCGTGG CCTTCACCTG
          · E  P  E   T  Q  D  A   T  Y  L   W  W  V   N  N  Q  S ·
501       CGAGCCCGAG ACCCAGGACG CCACCTACCT GTGGTGGGTG AACAACCAGA
          · L  P  V   S  P  R   L  Q  L  S   N  G  N   R  T  L
551       GCCTGCCCGT GAGCCCCCGC CTGCAGCTGA GCAACGGCAA CCGCACCCTG
           T  L  F  N   V  T  R   N  D  T   A  S  Y  K   C  E  T ·
601       ACCCTGTTCA ACGTGACCCG CAACGACACC GCCAGCTACA AGTGCGAGAC
          · Q  N  P   V  S  A  R   R  S  D   S  V  I   L  N  V  L ·
651       CCAGAACCCC GTGAGCGCCC GCCGCAGCGA CAGCGTGATC CTGAACGTGC
          · Y  G  P   D  A  P   T  I  S  P   L  N  T   S  Y  R
701       TGTACGGCCC CGACGCCCCC ACCATCAGCC CCCTGAACAC CAGCTACCGC
           S  G  E  N   L  N  L   S  C  H   A  A  S  N   P  P  A ·
751       AGCGGCGAGA ACCTGAACCT GAGCTGCCAC GCCGCCAGCA ACCCCCCCGC
          · Q  Y  S   W  F  V  N   G  T  F   Q  Q  S   T  Q  E  L ·
801       CCAGTACAGC TGGTTCGTGA ACGGCACCTT CCAGCAGAGC ACCCAGGAGC
          · F  I  P   N  I  T   V  N  N  S   G  S  Y   T  C  Q
851       TGTTCATCCC CAACATCACC GTGAACAACA GCGGCAGCTA CACCTGCCAG
           A  H  N  S   D  T  G   L  N  R   T  T  V  T   T  I  T ·
901       GCCCACAACA GCGACACCGG CCTGAACCGC ACCACCGTGA CCACCATCAC
          · V  Y  A   E  P  P  K   P  F  I   T  S  N   N  S  N  P ·
951       CGTGTACGCC GAGCCCCCCA AGCCCTTCAT CACCAGCAAC AACAGCAACC
          · V  E  D   E  D  A   V  A  L  T   C  E  P   E  I  Q
1001      CCGTGGAGGA CGAGGACGCC GTGGCCCTGA CCTGCGAGCC CGAGATCCAG
           N  T  T  Y   L  W  W   V  N  N   Q  S  L  P   V  S  P ·
1051      AACACCACCT ACCTGTGGTG GGTGAACAAC CAGAGCCTGC CCGTGAGCCC
          · R  L  Q   L  S  N  D   N  R  T   L  T  L   L  S  V  T ·
1101      CCGCCTGCAG CTGAGCAACG ACAACCGCAC CCTGACCCTG CTGAGCGTGA
          · R  N  D   V  G  P   Y  E  C  G   I  Q  N   E  L  S
1151      CCCGCAACGA CGTGGGCCCC TACGAGTGCG GCATCCAGAA CGAGCTGAGC
           V  D  H  S   D  P  V   I  L  N   V  L  Y  G   P  D  D ·
1201      GTGGACCACA GCGACCCCGT GATCCTGAAC GTGCTGTACG GCCCCGACGA
          · P  T  I   S  P  S  Y   T  Y  Y   R  P  G   V  N  L  S ·
1251      CCCCACCATC AGCCCCAGCT ACACCTACTA CCGCCCCGGC GTGAACCTGA
          · L  S  C   H  A  A   S  N  P  P   A  Q  Y   S  W  L
1301      GCCTGAGCTG CCACGCCGCC AGCAACCCCC CCGCCCAGTA CAGCTGGCTG
           I  D  G  N   I  Q  Q   H  T  Q   E  L  F  I   S  N  I ·
1351      ATCGACGGCA ACATCCAGCA GCACACCCAG GAGCTGTTCA TCAGCAACAT
          · T  E  K   N  S  G  L   Y  T  C   Q  A  N   N  S  A  S ·
1401      CACCGAGAAG AACAGCGGCC TGTACACCTG CCAGGCCAAC AACAGCGCCA
          · G  H  S   R  T  T   V  K  T  I   T  V  S   A  E  L
1451      GCGGCCACAG CCGCACCACC GTGAAGACCA TCACCGTGAG CGCCGAGCTG
```

```
          P  K  P  S  I  S  S  N  N  S  K  P  V  E  D  K  D ·
1501      CCCAAGCCCA GCATCAGCAG CAACAACAGC AAGCCCGTGG AGGACAAGGA
          ·  A  V  A  F  T  C  E  P  E  A  Q  N  T  T  Y  L  W ·
1551      CGCCGTGGCC TTCACCTGCG AGCCCGAGGC CCAGAACACC ACCTACCTGT
          ·  W  V  N  G  Q  S  L  P  V  S  P  R  L  Q  L  S
1601      GGTGGGTGAA CGGCCAGAGC CTGCCCGTGA GCCCCCGCCT GCAGCTGAGC
          N  G  N  R  T  L  T  L  F  N  V  T  R  N  D  A  R ·
1651      AACGGCAACC GCACCCTGAC CCTGTTCAAC GTGACCCGCA ACGACGCCCG
          ·  A  Y  V  C  G  I  Q  N  S  V  S  A  N  R  S  D  P ·
1701      CGCCTACGTG TGCGGCATCC AGAACAGCGT GAGCGCCAAC CGCAGCGACC
          ·  V  T  L  D  V  L  Y  G  P  D  T  P  I  I  S  P
1751      CCGTGACCCT GGACGTGCTG TACGGCCCCG ACACCCCCAT CATCAGCCCC
          P  D  S  S  Y  L  S  G  A  N  L  N  L  S  C  H  S ·
1801      CCCGACAGCA GCTACCTGAG CGGCGCCAAC CTGAACCTGA GCTGCCACAG
          ·  A  S  N  P  S  P  Q  Y  S  W  R  I  N  G  I  P  Q ·
1851      CGCCAGCAAC CCCAGCCCCC AGTACAGCTG GCGCATCAAC GGCATCCCCC
          ·  Q  H  T  Q  V  L  F  I  A  K  I  T  P  N  N  N
1901      AGCAGCACAC CCAGGTGCTG TTCATCGCCA AGATCACCCC CAACAACAAC
          G  T  Y  A  C  F  V  S  N  L  A  T  G  R  N  N  S ·
1951      GGCACCTACG CCTGCTTCGT GAGCAACCTG GCCACCGGCC GCAACAACAG
          ·  I  V  K  S  I  T  V  S  A  S  G  T  S  R  S  T  P ·
2001      CATCGTGAAG AGCATCACCG TGAGCGCCAG CGGCACCTCT AGAAGCACCC
          ·  I  P  F  S  Y  S  K  N  L  D  C  W  V  D  N  E
2051      CCATCCCATT CAGCTACAGC AAGAACCTGG ACTGCTGGGT GGACAACGAG
          E  D  I  D  V  I  L  K  K  S  T  I  L  N  L  D  I ·
2101      GAGGACATCG ACGTGATCCT GAAGAAGAGC ACCATCCTGA ACCTGGACAT
          ·  N  N  D  I  I  S  D  I  S  G  F  N  S  S  V  I  T ·
2151      CAACAACGAC ATCATCAGCG ACATCAGCGG CTTCAACAGC AGCGTGATCA
          ·  Y  P  D  A  Q  L  V  P  G  I  N  G  K  A  I  H
2201      CCTACCCCGA CGCCCAGCTG GTGCCCGGCA TCAACGGCAA GGCCATCCAC
          L  V  N  N  E  S  S  E  V  I  V  H  K  A  M  D  I ·
2251      CTGGTGAACA ACGAGAGCAG CGAGGTGATC GTGCACAAGG CCATGGACAT
          ·  E  Y  N  D  M  F  N  N  F  T  V  S  F  W  L  R  V ·
2301      CGAGTACAAC GACATGTTCA ACAACTTCAC CGTGAGCTTC TGGCTGAGAG
          ·  P  K  V  S  A  S  H  L  E  Q  Y  G  T  N  E  Y
2351      TGCCTAAGGT GAGCGCCAGC CACCTGGAGC AGTACGGCAC CAACGAGTAC
          S  I  I  S  S  M  K  K  H  S  L  S  I  G  S  G  W ·
2401      AGCATCATCA GCAGCATGAA GAAGCACAGC CTGAGCATCG GCAGCGGCTG
          ·  S  V  S  L  K  G  N  N  L  I  W  T  L  K  D  S  A ·
2451      GAGCGTGAGC CTGAAGGGCA ACAACCTCAT CTGGACCCTG AAGGATAGCG
          ·  G  E  V  R  Q  I  T  F  R  D  L  P  D  K  F  N
2501      CCGGAGAGGT GAGACAGATC ACCTTCAGAG ACCTGCCCGA CAAGTTCAAT
          A  Y  L  A  N  K  W  V  F  I  T  I  T  N  D  R  L ·
2551      GCCTACCTGG CCAACAAGTG GGTGTTCATC ACCATCACCA ACGACAGACT
          ·  S  S  A  N  L  Y  I  N  G  V  L  M  G  S  A  E  I ·
2601      GAGCAGCGCC AACCTGTACA TCAACGGCGT GCTCATGGGC AGCGCCGAGA
          ·  T  G  L  G  A  I  R  E  D  N  N  I  T  L  K  L
2651      TCACCGGCCT GGGCGCCATC AGAGAGGACA ACAACATCAC CCTGAAGCTG
          D  R  C  N  N  N  N  Q  Y  V  S  I  D  K  F  R  I ·
2701      GACAGATGCA ACAACAACAA CCAGTACGTG AGCATCGACA AGTTCCGGAT
          ·  F  C  K  A  L  N  P  K  E  I  E  K  L  Y  T  S  Y ·
2751      CTTCTGCAAG GCCCTGAACC CCAAGGAGAT CGAGAAGCTG TACACCAGCT
          ·  L  S  I  T  F  L  R  D  F  W  G  N  P  L  R  Y
2801      ACCTGAGCAT CACCTTCCTG AGAGACTTCT GGGGCAACCC CCTGAGATAC
          D  T  * (SEQ ID NO:1)
2851      GACACCTAG (SEQ ID NO:25)
```

FIGURE 9

```
        M  E  S  P  S  A  P  P  H  R  W  C  I  P  W  Q  R  L  L  L
   1 ATGGAGAGCC CCAGCGCCCC CCCCCACCGC TGGTGCATCC CCTGGCAGCG CCTGCTGCTG
        T  A  S  L  L  T  F  W  N  P  P  T  T  A  K  L  T  I  E  S
  61 ACCGCCAGCC TGCTGACCTT CTGGAACCCC CCCACCACCG CCAAGCTGAC CATCGAGAGC
        T  P  F  N  V  A  E  G  K  E  V  L  L  L  V  H  N  L  P  Q
 121 ACCCCCTTCA ACGTGGCCGA GGGCAAGGAG GTGCTGCTGC TGGTGCACAA CCTGCCCCAG
        H  L  F  G  Y  S  W  Y  K  G  E  R  V  D  G  N  R  Q  I  I
 181 CACCTGTTCG GCTACAGCTG GTACAAGGGC GAGCGCGTGG ACGGCAACCG CCAGATCATC
        G  Y  V  I  G  T  Q  Q  A  T  P  G  P  A  Y  S  G  R  E  I
 241 GGCTACGTGA TCGGCACCCA GCAGGCCACC CCCGGCCCCG CCTACAGCGG CCGCGAGATC
        I  Y  P  N  A  S  L  L  I  Q  N  I  I  Q  N  D  T  G  F  Y
 301 ATCTACCCCA ACGCCAGCCT GCTGATCCAG AACATCATCC AGAACGACAC CGGCTTCTAC
        T  L  H  V  I  K  S  D  L  V  N  E  E  A  T  G  Q  F  R  V
 361 ACCCTGCACG TGATCAAGAG CGACCTGGTG AACGAGGAGG CCACCGGCCA GTTCCGCGTG
        Y  P  E  L  P  K  P  S  I  S  S  N  N  S  K  P  V  E  D  K
 421 TACCCCGAGC TGCCCAAGCC CAGCATCAGC AGCAACAACA GCAAGCCCGT GGAGGACAAG
        D  A  V  A  F  T  C  E  P  E  T  Q  D  A  T  Y  L  W  W  V
 481 GACGCCGTGG CCTTCACCTG CGAGCCCGAG ACCCAGGACG CCACCTACCT GTGGTGGGTG
        N  N  Q  S  L  P  V  S  P  R  L  Q  L  S  N  G  N  R  T  L
 541 AACAACCAGA GCCTGCCCGT GAGCCCCCGC CTGCAGCTGA GCAACGGCAA CCGCACCCTG
        T  L  F  N  V  T  R  N  D  T  A  S  Y  K  C  E  T  Q  N  P
 601 ACCCTGTTCA ACGTGACCCG CAACGACACC GCCAGCTACA AGTGCGAGAC CCAGAACCCC
        V  S  A  R  R  S  D  S  V  I  L  N  V  L  Y  G  P  D  A  P
 661 GTGAGCGCCC GCCGCAGCGA CAGCGTGATC CTGAACGTGC TGTACGGCCC CGACGCCCCC
        T  I  S  P  L  N  T  S  Y  R  S  G  E  N  L  N  L  S  C  H
 721 ACCATCAGCC CCCTGAACAC CAGCTACCGC AGCGGCGAGA ACCTGAACCT GAGCTGCCAC
        A  A  S  N  P  P  A  Q  Y  S  W  F  V  N  G  T  F  Q  Q  S
 781 GCCGCCAGCA ACCCCCCCGC CCAGTACAGC TGGTTCGTGA ACGGCACCTT CCAGCAGAGC
        T  Q  E  L  F  I  P  N  I  T  V  N  N  S  G  S  Y  T  C  Q
 841 ACCCAGGAGC TGTTCATCCC CAACATCACC GTGAACAACA GCGGCAGCTA CACCTGCCAG
        A  H  N  S  D  T  G  L  N  R  T  T  V  T  T  I  T  V  Y  A
 901 GCCCACAACA GCGACACCGG CCTGAACCGC ACCACCGTGA CCACCATCAC CGTGTACGCC
        E  P  P  K  P  F  I  T  S  N  N  S  N  P  V  E  D  E  D  A
 961 GAGCCCCCCA AGCCCTTCAT CACCAGCAAC AACAGCAACC CCGTGGAGGA CGAGGACGCC
        V  A  L  T  C  E  P  E  I  Q  N  T  T  Y  L  W  W  V  N  N
1021 GTGGCCCTGA CCTGCGAGCC CGAGATCCAG AACACCACCT ACCTGTGGTG GGTGAACAAC
        Q  S  L  P  V  S  P  R  L  Q  L  S  N  D  N  R  T  L  T  L
1081 CAGAGCCTGC CCGTGAGCCC CCGCCTGCAG CTGAGCAACG ACAACCGCAC CCTGACCCTG
        L  S  V  T  R  N  D  V  G  P  Y  E  C  G  I  Q  N  E  L  S
1141 CTGAGCGTGA CCCGCAACGA CGTGGGCCCC TACGAGTGCG GCATCCAGAA CGAGCTGAGC
        V  D  H  S  D  P  V  I  L  N  V  L  Y  G  P  D  D  P  T  I
1201 GTGGACCACA GCGACCCCGT GATCCTGAAC GTGCTGTACG GCCCCGACGA CCCCACCATC
        S  P  S  Y  T  Y  Y  R  P  G  V  N  L  S  L  S  C  H  A  A
1261 AGCCCCAGCT ACACCTACTA CCGCCCCGGC GTGAACCTGA GCCTGAGCTG CCACGCCGCC
        S  N  P  P  A  Q  Y  S  W  L  I  D  G  N  I  Q  Q  H  T  Q
1321 AGCAACCCCC CCGCCCAGTA CAGCTGGCTG ATCGACGGCA ACATCCAGCA GCACACCCAG
        E  L  F  I  S  N  I  T  E  K  N  S  G  L  Y  T  C  Q  A  N
1381 GAGCTGTTCA TCAGCAACAT CACCGAGAAG AACAGCGGCC TGTACACCTG CCAGGCCAAC
        N  S  A  S  G  H  S  R  T  T  V  K  T  I  T  V  S  A  E  L
1441 AACAGCGCCA GCGGCCACAG CCGCACCACC GTGAAGACCA TCACCGTGAG CGCCGAGCTG
        P  K  P  S  I  S  S  N  N  S  K  P  V  E  D  K  D  A  V  A
1501 CCCAAGCCCA GCATCAGCAG CAACAACAGC AAGCCCGTGG AGGACAAGGA CGCCGTGGCC
        F  T  C  E  P  E  A  Q  N  T  T  Y  L  W  W  V  N  G  Q  S
1561 TTCACCTGCG AGCCCGAGGC CCAGAACACC ACCTACCTGT GGTGGGTGAA CGGCCAGAGC
        L  P  V  S  P  R  L  Q  L  S  N  G  N  R  T  L  T  L  F  N
1621 CTGCCCGTGA GCCCCCGCCT GCAGCTGAGC AACGGCAACC GCACCCTGAC CCTGTTCAAC
        V  T  R  N  D  A  R  A  Y  V  C  G  I  Q  N  S  V  S  A  N
1681 GTGACCCGCA ACGACGCCCG CGCCTACGTG TGCGGCATCC AGAACAGCGT GAGCGCCAAC
        R  S  D  P  V  T  L  D  V  L  Y  G  P  D  T  P  I  I  S  P
1741 CGCAGCGACC CCGTGACCCT GGACGTGCTG TACGGCCCCG ACACCCCCAT CATCAGCCCC
```

```
          P  D  S  S  Y  L  S     G  A  N     L  N  L  S     C  H  S     A  S  N
1801  CCCGACAGCA GCTACCTGAG CGGCGCCAAC CTGAACCTGA GCTGCCACAG CGCCAGCAAC
          P  S  P  Q  Y  S  W     R  I  N     G  I  P  Q     Q  H  T     Q  V  L
1861  CCCAGCCCCC AGTACAGCTG GCGCATCAAC GGCATCCCCC AGCAGCACAC CCAGGTGCTG
          F  I  A  K  I  T  P     N  N  N     G  T  Y  A     C  F  V     S  N  L
1921  TTCATCGCCA AGATCACCCC CAACAACAAC GGCACCTACG CCTGCTTCGT GAGCAACCTG
          A  T  G  R  N  N  S     I  V  K     S  I  T  V     S  A  S     G  T  S
1981  GCCACCGGCC GCAACAACAG CATCGTGAAG AGCATCACCG TGAGCGCCAG CGGCACCTCT
          R  K  T  H  T  C  P     P  C  P     A  P  E  L     L  G  G     P  S  V
2041  AGAAAGACCC ACACCTGCCC CCCTTGCCCT GCCCCTGAGC TGCTGGGCGG ACCCAGCGTG
          F  L  F  P  P  K  P     K  D  T     L  M  I  S     R  T  P     E  V  T
2101  TTCCTGTTCC CCCCCAAGCC TAAGGACACC CTCATGATCA GCAGAACCCC CGAGGTGACC
          C  V  V  V  D  V  S     H  E  D     P  E  V  K     F  N  W     Y  V  D
2161  TGCGTGGTGG TGGACGTGAG CCACGAGGAT CCCGAGGTGA AGTTCAACTG GTACGTGGAC
          G  V  E  V  H  N  A     K  T  K     P  R  E  E     Q  Y  N     S  T  Y
2221  GGCGTGGAGG TGCACAATGC CAAGACCAAG CCCAGAGAGG AGCAGTACAA CAGCACCTAC
          R  V  V  S  V  L  T     V  L  H     Q  D  W  L     N  G  K     E  Y  K
2281  AGAGTGGTGA GCGTGCTCAC CGTGCTGCAC CAGGATTGGC TGAACGGCAA GGAGTACAAG
          C  K  V  S  N  K  A     L  P  A     P  I  E  K     T  I  S     K  A  K
2341  TGCAAGGTGA GCAACAAGGC CCTGCCTGCC CCCATCGAGA AAACCATCAG CAAGGCCAAG
          G  Q  P  R  E  P  Q     V  Y  T     L  P  P  S     R  D  E     L  T  K
2401  GGCCAGCCCA GAGAGCCCCA GGTGTACACC CTGCCCCCTA GCAGAGATGA GTTGACCAAG
          N  Q  V  S  L  T  C     L  V  K     G  F  Y  P     S  D  I     A  V  E
2461  AACCAGGTGA GCCTCACATG CCTGGTGAAG GGCTTCTACC CCAGCGACAT CGCCGTGGAG
          W  E  S  N  G  Q  P     E  N  N     Y  K  T  T     P  P  V     L  D  S
2521  TGGGAGAGCA ACGGCCAGCC CGAGAACAAC TACAAGACCA CCCCCCCTGT GCTGGACAGC
          D  G  S  F  F  L  Y     S  K  L     T  V  D  K     S  R  W     Q  Q  G
2581  GATGGCAGCT TCTTCCTGTA CAGCAAGCTC ACCGTGGACA AGAGCAGATG GCAGCAGGGC
          N  V  F  S  C  S  V     M  H  E     A  L  H  N     H  Y  T     Q  K  S
2641  AACGTGTTCA GCTGCAGCGT GATGCACGAG GCCCTGCACA ATCACTACAC CCAGAAGAGC
          L  S  L  S  P  G  K     *  (SEQ ID NO:26)
2701  CTGAGCCTGA GCCCCGGCAA GTAA     (SEQ ID NO:5)
```

FIGURE 10

A.

```
atggagtctc cctcggcccc tccccacaga tggtgcatcc cctggcagag gctcctgctc 60
acagcctcac ttctaacctt ctggaacccg cccaccactg ccaagctcac tattgaatcc 120
acgccgttca atgtcgcaga ggggaaggag gtgcttctac ttgtccacaa tctgccccag 180
catcttttg gctacagctg gtacaaaggt gaaagagtgg atggcaaccg tcaaattata 240
ggatatgtaa taggaactca acaagctacc ccagggcccg catacagtgg tcgagagata 300
atatacccca atgcatccct gctgatccag aacatcatcc agaatgacac aggattctac 360
accctacacg tcataaagtc agatcttgtg aatgaagaag caactggcca gttccgggta 420
tacccggagc tgcccaagcc ctccatctcc agcaacaact ccaaacccgt ggaggacaag 480
gatgctgtgg ccttcacctg tgaacctgag actcaggacg caacctacct gtggtgggta 540
aacaatcaga gcctcccggt cagtcccagg ctgcagctgt ccaatggcaa caggaccctc 600
actctattca atgtcacaag aaatgacaca gcaagctaca aatgtgaaac ccagaaccca 660
gtgagtgcca ggcgcagtga ttcagtcatc ctgaatgtcc tctatggccc ggatgccccc 720
accatttccc ctctaaacac atcttacaga tcaggggaaa atctgaacct ctcctgccac 780
gcagcctcta acccacctgc acagtactct tggtttgtca atgggacttt ccagcaatcc 840
acccaagagc tctttatccc caacatcact gtgaataata gtggatccta tacgtgccaa 900
gcccataact cagacactgg cctcaatagg accacagtca cgacgatcac agtctatgca 960
gagccaccca aacccttcat caccagcaac aactccaacc ccgtggagga tgaggatgct 1020
gtagccttaa cctgtgaacc tgagattcag aacacaacct acctgtggtg ggtaaataat 1080
cagagcctcc cggtcagtcc caggctgcag ctgtccaatg acaacaggac cctcactcta 1140
ctcagtgtca caaggaatga tgtaggaccc tatgagtgtg gaatccagaa cgaattaagt 1200
gttgaccaca gcgacccagt catcctgaat gtcctctatg gcccagacga ccccaccatt 1260
tccccctcat acacctatta ccgtccaggg gtgaacctca gcctctcctg ccatgcagcc 1320
tctaacccac ctgcacagta ttcttggctg attgatggga acatccagca acacacacaa 1380
gagctcttta tctccaacat cactgagaag aacagcggac tctatacctg ccaggccaat 1440
aactcagcca gtggccacag caggactaca gtcaagacaa tcacagtctc tgcggagctg 1500
cccaagccct ccatctccag caacaactcc aaacccgtgg aggacaagga tgctgtggcc 1560
ttcacctgtg aacctgaggc tcagaacaca acctacctgt ggtgggtaaa tggtcagagc 1620
ctcccagtca gtcccaggct gcagctgtcc aatggcaaca ggaccctcac tctattcaat 1680
gtcacaagaa atgacgcaag agcctatgta tgtggaatcc agaactcagt gagtgcaaac 1740
cgcagtgacc cagtcaccct ggatgtcctc tatgggccgg acacccccat catttccccc 1800
ccagactcgt cttacctttc gggagcgaac ctcaacctct cctgccactc ggcctctaac 1860
ccatccccgc agtattcttg gcgtatcaat gggataccgc agcaacacac acaagttctc 1920
tttatcgcca aaatcacgcc aaataataac gggacctatg cctgttttgt ctctaacttg 1980
gctactggcc gcaataattc catagtcaag agcatcacag tctctgcatc tggaact (SEQ ID NO:2)
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B.

```
   1  MESPSAPPHR WCIPWQRLLL TASLLTFWNP PTTAKLTIES TPFNVAEGKE
  51  VLLLVHNLPQ HLFGYSWYKG ERVDGNRQII GYVIGTQQAT PGPAYSGREI
 101  IYPNASLLIQ NIIQNDTGFY TLHVIKSDLV NEEATGQFRV YPELPKPSIS
 151  SNNSKPVEDK DAVAFTCEPE TQDATYLWWV NNQSLPVSPR LQLSNGNRTL
 201  TLFNVTRNDT ASYKCETQNP VSARRSDSVI LNVLYGPDAP TISPLNTSYR
 251  SGENLNLSCH AASNPPAQYS WFVNGTFQQS TQELFIPNIT VNNSGSYTCQ
 301  AHNSDTGLNR TTVTTITVYA EPPKPFITSN NSNPVEDEDA VALTCEPEIQ
 351  NTTYLWWVNN QSLPVSPRLQ LSNDNRTLTL LSVTRNDVGP YECGIQNELS
 401  VDHSDPVILN VLYGPDDPTI SPSYTYYRPG VNLSLSCHAA SNPPAQYSWL
 451  IDGNIQQHTQ ELFISNITEK NSGLYTCQAN NSASGHSRTT VKTITVSAEL
 501  PKPSISSNNS KPVEDKDAVA FTCEPEAQNT TYLWWVNGQS LPVSPRLQLS
 551  NGNRTLTLFN VTRNDARAYV CGIQNSVSAN RSDPVTLDVL YGPDTPIISP
 601  PDSSYLSGAN LNLSCHSASN PSPQYSWRIN GIPQQHTQVL FIAKITPNNN
 651  GTYACFVSNL ATGRNNSIVK SITVSASGT (SEQ ID NO:3)
```

FIGURE 11

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      D  S  T  P  I  P  F     S  Y  S     K  N  L  D     C  W  V     D  N  E
  1   GATTCAACAC CAATTCCATT TTCTTATTCT AAAAATCTGG ATTGTTGGGT TGATAATGAA
      E  D  I  D  V  I  L     K  K  S     T  I  L  N     L  D  I     N  N  D
 61   GAAGATATAG ATGTTATATT AAAAAAGAGT ACAATTTTAA ATTTAGATAT TAATAATGAT
      I  I  S  D  I  S  G     F  N  S     S  V  I  T     Y  P  D     A  Q  L
121   ATTATATCAG ATATATCTGG GTTTAATTCA TCTGTAATAA CATATCCAGA TGCTCAATTG
      V  P  G  I  N  G  K     A  I  H     L  V  N  N     E  S  S     E  V  I
181   GTGCCCGGAA TAAATGGCAA AGCAATACAT TTAGTAAACA ATGAATCTTC TGAAGTTATA
      V  H  K  A  M  D  I     E  Y  N     D  M  F  N     N  F  T     V  S  F
241   GTGCATAAAG CTATGGATAT TGAATATAAT GATATGTTTA ATAATTTTAC CGTTAGCTTT
      W  L  R  V  P  K  V     S  A  S     H  L  E  Q     Y  G  T     N  E  Y
301   TGGTTGAGGG TTCCTAAAGT ATCTGCTAGT CATTTAGAAC AATATGGCAC AAATGAGTAT
      S  I  I  S  S  M  K     K  H  S     L  S  I  G     S  G  W     S  V  S
361   TCAATAATTA GCTCTATGAA AAAACATAGT CTATCAATAG GATCTGGTTG GAGTGTATCA
      L  K  G  N  N  L  I     W  T  L     K  D  S  A     G  E  V     R  Q  I
421   CTTAAAGGTA ATAACTTAAT ATGGACTTTA AAAGATTCCG CGGGAGAAGT TAGACAAATA
      T  F  R  D  L  P  D     K  F  N     A  Y  L  A     N  K  W     V  F  I
481   ACTTTTAGGG ATTTACCTGA TAAATTTAAT GCTTATTTAG CAAATAAATG GGTTTTTATA
      T  I  T  N  D  R  L     S  S  A     N  L  Y  I     N  G  V     L  M  G
541   ACTATTACTA ATGATAGATT ATCTTCTGCT AATTTGTATA TAAATGGAGT ACTTATGGGA
      S  A  E  I  T  G  L     G  A  I     R  E  D  N     N  I  T     L  K  L
601   AGTGCAGAAA TTACTGGTTT AGGAGCTATT AGAGAGGATA ATAATATAAC ATTAAAACTA
      D  R  C  N  N  N  N     Q  Y  V     S  I  D  K     F  R  I     F  C  K
661   GATAGATGTA ATAATAATAA TCAATACGTT TCTATTGATA AATTTAGGAT ATTTTGCAAA
      A  L  N  P  K  E  I     E  K  L     Y  T  S  Y     L  S  I     T  F  L
721   GCATTAAATC CAAAAGAGAT TGAAAAATTA TACACAAGTT ATTTATCTAT AACCTTTTTA
      R  D  F  W  G  N  P     L  R  Y     D  T  D  R     *(SEQ ID NO:28)
781   AGAGACTTCT GGGGAAACCC TTTACGATAT GATACAGATA GGTAG (SEQ ID NO:27)
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FIGURE 12

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   1    ATGGAGTCTC CCTCGGCCCC TCCCCACAGA TGGTGCATCC CCTGGCAGAG GCTCCTGCTC
  61    ACAGCCTCAC TTCTAACCTT CTGGAACCCG CCCACCACTG CCAAGCTCAC TATTGAATCC
 121    ACGCCGTTCA ATGTCGCAGA GGGGAAGGAG GTGCTTCTAC TTGTCCACAA TCTGCCCCAG
 181    CATCTTTTTG GCTACAGCTG GTACAAAGGT GAAAGAGTGG ATGGCAACCG TCAAATTATA
 241    GGATATGTAA TAGGAACTCA ACAAGCTACC CCAGGGCCCG CATACAGTGG TCGAGAGATA
 301    ATATACCCCA ATGCATCCCT GCTGATCCAG AACATCATCC AGAATGACAC AGGATTCTAC
 361    ACCCTACACG TCATAAAGTC AGATCTTGTG AATGAAGAAG CAACTGGCCA GTTCCGGGTA
 421    TACCCGGAGC TGCCCAAGCC CTCCATCTCC AGCAACAACT CCAAACCCGT GGAGGACAAG
 481    GATGCTGTGG CCTTCACCTG TGAACCTGAG ACTCAGGACG CAACCTACCT GTGGTGGGTA
 541    AACAATCAGA GCCTCCCGGT CAGTCCCAGG CTGCAGCTGT CCAATGGCAA CAGGACCCTC
 601    ACTCTATTCA ATGTCACAAG AAATGACACA GCAAGCTACA AATGTGAAAC CCAGAACCCA
 661    GTGAGTGCCA GGCGCAGTGA TTCAGTCATC CTGAATGTCC TCTATGGCCC GGATGCCCCC
 721    ACCATTTCCC CTCTAAACAC ATCTTACAGA TCAGGGGAAA ATCTGAACCT CTCCTGCCAC
 781    GCAGCCTCTA ACCCACCTGC ACAGTACTCT TGGTTTGTCA ATGGGACTTT CCAGCAATCC
 841    ACCCAAGAGC TCTTTATCCC CAACATCACT GTGAATAATA GTGGATCCTA TACGTGCCAA
 901    GCCCATAACT CAGACACTGG CCTCAATAGG ACCACAGTCA CGACGATCAC AGTCTATGCA
 961    GAGCCACCCA AACCCTTCAT CACCAGCAAC AACTCCAACC CCGTGGAGGA TGAGGATGCT
1021    GTAGCCTTAA CCTGTGAACC TGAGATTCAG AACACAACCT ACCTGTGGTG GGTAAATAAT
1081    CAGAGCCTCC CGGTCAGTCC CAGGCTGCAG CTGTCCAATG ACAACAGGAC CCTCACTCTA
1141    CTCAGTGTCA CAAGGAATGA TGTAGGACCC TATGAGTGTG GAATCCAGAA CGAATTAAGT
1201    GTTGACCACA GCGACCCAGT CATCCTGAAT GTCCTCTATG GCCCAGACGA CCCCACCATT
1261    TCCCCCTCAT ACACCTATTA CCGTCCAGGG GTGAACCTCA GCCTCTCCTG CCATGCAGCC
1321    TCTAACCCAC CTGCACAGTA TTCTTGGCTG ATTGATGGGA ACATCCAGCA ACACACACAA
1381    GAGCTCTTTA TCTCCAACAT CACTGAGAAG AACAGCGGAC TCTATACCTG CCAGGCCAAT
1441    AACTCAGCCA GTGGCCACAG CAGGACTACA GTCAAGACAA TCACAGTCTC TGCGGAGCTG
1501    CCCAAGCCCT CCATCTCCAG CAACAACTCC AAACCCGTGG AGGACAAGGA TGCTGTGGCC
1561    TTCACCTGTG AACCTGAGGC TCAGAACACA ACCTACCTGT GGTGGGTAAA TGGTCAGAGC
1621    CTCCCAGTCA GTCCCAGGCT GCAGCTGTCC AATGGCAACA GGACCCTCAC TCTATTCAAT
1681    GTCACAAGAA ATGACGCAAG AGCCTATGTA TGTGGAATCC AGAACTCAGT GAGTGCAAAC
1741    CGCAGTGACC CAGTCACCCT GGATGTCCTC TATGGGCCGG ACACCCCCAT CATTTCCCCC
1801    CCAGACTCGT CTTACCTTTC GGGAGCGAAC CTCAACCTCT CCTGCCACTC GGCCTCTAAC
1861    CCATCCCCGC AGTATTCTTG GCGTATCAAT GGGATACCGC AGCAACACAC ACAAGTTCTC
1921    TTTATCGCCA AAATCACGCC AAATAATAAC GGGACCTATG CCTGTTTTGT CTCTAACTTG
1981    GCTACTGGCC GCAATAATTC CATAGTCAAG AGCATCACAG TCTCTGCATC TGGAACTCTA
2041    GATTCAACAC CAATTCCATT TTCTTATTCT AAAAATCTGG ATTGTTGGGT TGATAATGAA
2101    GAAGATATAG ATGTTATATT AAAAAAGAGT ACAATTTTAA ATTTAGATAT TAATAATGAT
2161    ATTATATCAG ATATATCTGG GTTTAATTCA TCTGTAATAA CATATCCAGA TGCTCAATTG
2221    GTGCCCGGAA TAAATGGCAA AGCAATACAT TTAGTAAACA ATGAATCTTC TGAAGTTATA
2281    GTGCATAAAG CTATGGATAT TGAATATAAT GATATGTTTA ATAATTTTAC CGTTAGCTTT
2341    TGGTTGAGGG TTCCTAAAGT ATCTGCTAGT CATTTAGAAC AATATGGCAC AAATGAGTAT
2401    TCAATAATTA GCTCTATGAA AAAACATAGT CTATCAATAG GATCTGGTTG GAGTGTATCA
2461    CTTAAAGGTA ATAACTTAAT ATGGACTTTA AAAGATTCCG CGGGAGAAGT TAGACAAATA
2521    ACTTTTAGGG ATTTACCTGA TAAATTTAAT GCTTATTTAG CAAATAAATG GGTTTTTATA
2581    ACTATTACTA ATGATAGATT ATCTTCTGCT AATTTGTATA TAAATGGAGT ACTTATGGGA
2641    AGTGCAGAAA TTACTGGTTT AGGAGCTATT AGAGAGGATA ATAATATAAC ATTAAAACTA
2701    GATAGATGTA ATAATAATAA TCAATACGTT TCTATTGATA AATTTAGGAT ATTTTGCAAA
2761    GCATTAAATC CAAAAGAGAT TGAAAAATTA TACACAAGTT ATTTATCTAT AACCTTTTTA
2821    AGAGACTTCT GGGGAAACCC TTTACGATAT GATATAG (SEQ ID NO:29)
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FIGURE 13

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   1  ATGGGGTCTC CCTCAGCCCC TCTTCACAGA TGGTGCATCC CCTGGCAGAC
  51  GCTCCTGCTC ACAGCCTCAC TTCTAACCTT CTGGAACCCG CCCACCACTG
 101  CCCAGCTCAC TATTGAATCC AGGCCGTTCA ATGTTGCAGA GGGGAAGGAG
 151  GTTCTTCTAC TTGCCCACAA TGTGTCCCAG AATCTTTTTG GCTACATTTG
 201  GTACAAGGGA GAAAGAGTGG ATGCCAGCCG TCGAATTGGA TCATGTGTAA
 251  TAAGAACTCA ACAAATTACC CCAGGGCCCG CACACAGCGG TCGAGAGACA
 301  ATAGACTTCA ATGCATCCCT GCTGATCCAC AATGTCACCC AGAGTGACAC
 351  AGGATCCTAC ACCATACAAG TCATAAAGGA AGATCTTGTG AATGAAGAAG
 401  CAACTGGCCA GTTCCGGGTA TACCCGGAGC TGCCCAAGCC CTACATCTCC
 451  AGCAACAACT CCAACCCCGT GGAGGACAAG GATGCTGTGG CCTTAACCTG
 501  TGAACCTGAG ACTCAGGACA CAACCTACCT GTGGTGGGTA AACAATCAGA
 551  GCCTCCCGGT CAGTCCCAGG CTGGAGCTGT CCAGTGACAA CAGGACCCTC
 601  ACTGTATTCA ATATTCCAAG AAATGACACA ACATCCTACA AATGTGAAAC
 651  CCAGAACCCA GTGAGTGTCA GACGCAGCGA CCCAGTCACC CTGAACGTCC
 701  TCTATGGCCC GGATGCGCCC ACCATTTCCC CTCTAAACAC ACCTTACAGA
 751  GCAGGGGAAA ATCTGAACCT CACCTGCCAC GCAGCCTCTA ACCCAACTGC
 801  ACAGTACTTT TGGTTTGTCA ATGGGACGTT CCAGCAATCC ACACAAGAGC
 851  TCTTTATACC CAACATCACC GTGAATAATA GCGGATCCTA TATGTGCCAA
 901  GCCCATAACT CAGCCACTGG CCTCAATAGG ACCACAGTCA CGGCGATCAC
 951  AGTCTACGCG GAGCTGCCCA AGCCCTACAT CACCAGCAAC AACTCCAACC
1001  CCATAGAGGA CAAGGATGCT GTGACCTTAA CCTGTGAACC TGAGACTCAG
1051  GACACAACCT ACCTGTGGTG GGTAAACAAT CAGAGCCTCT CGGTCAGTTC
1101  CAGGCTGGAG CTGTCCAATG ACAACAGGAC CCTCACTGTA TTCAATATTC
1151  CAAGAAACGA CACAACGTTC TACGAATGTG AGACCCAGAA CCCAGTGAGT
1201  GTCAGACGCA GCGACCCAGT CACCCTGAAT GTCCTCTATG GCCCGGATGC
1251  GCCCACCATT TCCCCTCTAA ACACACCTTA CAGAGCAGGG GAAAATCTGA
1301  ACCTCTCCTG CCACGCAGCC TCTAACCCAG CTGCACAGTA CTCTTGGTTT
1351  GTCAATGGGA CGTTCCAGCA ATCCACACAA GAGCTCTTTA TACCCAACAT
1401  CACCGTGAAT AATAGCGGAT CCTATATGTG CCAAGCCCAT AACTCAGCCA
1451  CTGGCCTCAA TAGGACCACA GTCACGGCGA TCACAGTCTA TGTGGAGCTG
1501  CCCAAGCCCT ACATCTCCAG CAACAACTCC AACCCCATAG AGGACAAGGA
1551  TGCTGTGACC TTAACCTGTG AACCTGTGGC TGAGAACACA ACCTACCTGT
1601  GGTGGGTAAA CAATCAGAGC CTCTCGGTCA GTCCCAGGCT GCAGCTCTCC
1651  AATGGCAACA GGATCCTCAC TCTACTCAGT GTCACACGGA ATGACACAGG
1701  ACCCTATGAA TGTGGAATCC AGAACTCAGA GAGTGCAAAA CGCAGTGACC
1751  CAGTCACCCT GAATGTCACC TATGGCCCAG ACACCCCCAT CATATCCCCC
1801  CCAGACTTGT CTTACCGTTC GGGAGCAAAC CTCAACCTCT CCTGCCACTC
1851  GGACTCTAAC CCATCCCCGC AGTATTCTTG GCTTATCAAT GGGACACTGC
1901  GGCAACACAC ACAAGTTCTC TTTATCTCCA AAATCACATC AAACAATAGC
1951  GGGGCCTATG CCTGTTTTGT CTCTAACTTG GCTACCGGTC GCAATAACTC
2001  CATAGTCAAG AACATCTCAG TCTCCTCTGG CGATTCAGCA CCTGGAAGTT
2051  CTGGTCTCTC AGCTAGGGCT ACTGTCGGCA TCATAATTGG AATGCTGGTT
2101  GGGGTTGCTC TGATGTAG (SEQ ID NO:31)
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FIGURE 14A

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   1    ATGGGGTCTC CCTCAGCCCC TCTTCACAGA TGGTGCATCC CCTGGCAGAC
  51    GCTCCTGCTC ACAGCCTCAC TTCTAACCTT CTGGAACCCG CCCACCACTG
 101    CCCAGCTCAC TATTGAATCC AGGCCGTTCA ATGTTGCAGA GGGGAAGGAG
 151    GTTCTTCTAC TTGCCCACAA TGTGTCCCAG AATCTTTTTG GCTACATTTG
 201    GTACAAGGGA GAAAGAGTGG ATGCCAGCCG TCGAATTGGA TCATGTGTAA
 251    TAAGAACTCA ACAAATTACC CCAGGGCCCG CACACAGCGG TCGAGAGACA
 301    ATAGACTTCA ATGCATCCCT GCTGATCCAC AATGTCACCC AGAGTGACAC
 351    AGGATCCTAC ACCATACAAG TCATAAAGGA AGATCTTGTG AATGAAGAAG
 401    CAACTGGCCA GTTCCGGGTA TACCCGGAGC TGCCCAAGCC CTACATCTCC
 451    AGCAACAACT CCAACCCCGT GGAGGACAAG GATGCTGTGG CCTTAACCTG
 501    TGAACCTGAG ACTCAGGACA CAACCTACCT GTGGTGGGTA AACAATCAGA
 551    GCCTCCCGGT CAGTCCCAGG CTGGAGCTGT CCAGTGACAA CAGGACCCTC
 601    ACTGTATTCA ATATTCCAAG AAATGACACA ACATCCTACA AATGTGAAAC
 651    CCAGAACCCA GTGAGTGTCA GACGCAGCGA CCCAGTCACC CTGAACGTCC
 701    TCTATGGCCC GGATGCGCCC ACCATTTCCC CTCTAAACAC ACCTTACAGA
 751    GCAGGGGAAA ATCTGAACCT CACCTGCCAC GCAGCCTCTA ACCCAACTGC
 801    ACAGTACTTT TGGTTTGTCA ATGGGACGTT CCAGCAATCC ACACAAGAGC
 851    TCTTTATACC CAACATCACC GTGAATAATA GCGGATCCTA TATGTGCCAA
 901    GCCCATAACT CAGCCACTGG CCTCAATAGG ACCACAGTCA CGGCGATCAC
 951    AGTCTACGCG GAGCTGCCCA AGCCCTACAT CACCAGCAAC AACTCCAACC
1001    CCATAGAGGA CAAGGATGCT GTGACCTTAA CCTGTGAACC TGAGACTCAG
1051    GACACAACCT ACCTGTGGTG GGTAAACAAT CAGAGCCTCT CGGTCAGTTC
1101    CAGGCTGGAG CTGTCCAATG ACAACAGGAC CCTCACTGTA TTCAATATTC
1151    CAAGAAACGA CACAACGTTC TACGAATGTG AGACCCAGAA CCCAGTGAGT
1201    GTCAGACGCA GCGACCCAGT CACCCTGAAT GTCCTCTATG GCCCGGATGC
1251    GCCCACCATT TCCCCTCTAA ACACACCTTA CAGAGCAGGG GAAAATCTGA
1301    ACCTCTCCTG CCACGCAGCC TCTAACCCAG CTGCACAGTA CTTTTGGTTT
1351    GTCAATGGGA CGTTCCAGCA ATCCACACAA GAGCTCTTTA TACCCAACAT
1401    CACCGTGAAT AATAGCGGAT CCTATATGTG CCAAGCCCAT AACTCAGCCA
1451    CTGGCCTCAA TAGGACCACA GTCACGGCGA TCACAGTCTA TGTGGAGCTG
1501    CCCAAGCCCT ACATCTCCAG CAACAACTCC AACCCCATAG AGGACAAGGA
1551    TGCTGTGACC TTAACCTGTG AACCTGTGGC TGAGAACACA ACCTACCTGT
1601    GGTGGGTAAA CAATCAGAGC CTCTCGGTCA GTCCCAGGCT GCAGCTCTCC
1651    AATGGCAACA GGATCCTCAC TCTACTCAGT GTCACACGGA ATGACACAGG
1701    ACCCTATGAA TGTGGAATCC AGAACTCAGA GAGTGCAAAA CGCAGTGACC
1751    CAGTCACCCT GAATGTCACC TATGGCCCAG ACACCCCCAT CATATCCCCC
1801    CCAGACTTGT CTTACCGTTC GGGAGCAAAC CTCAACCTCT CCTGCCACTC
1851    GGACTCTAAC CCATCCCCGC AGTATTCTTG GCTTATCAAT GGGACACTGC
1901    GGCAACACAC ACAAGTTCTC TTTATCTCCA AAATCACATC AAACAATAAC
1951    GGGGCCTATG CCTGTTTTGT CTCTAACTTG GCTACCGGTC GCAATAACTC
2001    CATAGTCAAG AACATCTCAG TCTCCTCTGG CGATTCAGCA CCTGGAAGTT
2051    CTGGTCTCTC AGCTAGGGCT ACTGTCGGCA TCATAATTGG AATGCTGGTT
2101    GGGGTTGCTC TGATGTAG (SEQ ID NO:32)
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FIGURE 14B

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  1    MGSPSAPLHR WCIPWQTLLL TASLLTFWNP PTTAQLTIES RPFNVAEGKE
 51    VLLLAHNVSQ NLFGYIWYKG ERVDASRRIG SCVIRTQQIT PGPAHSGRET
101    IDFNASLLIH NVTQSDTGSY TIQVIKEDLV NEEATGQFRV YPELPKPYIS
151    SNNSNPVEDK DAVALTCEPE TQDTTYLWWV NNQSLPVSPR LELSSDNRTL
201    TVFNIPRNDT TSYKCETQNP VSVRRSDPVT LNVLYGPDAP TISPLNTPYR
251    AGENLNLTCH AASNPTAQYF WFVNGTFQQS TQELFIPNIT VNNSGSYMCQ
301    AHNSATGLNR TTVTAITVYA ELPKPYITSN NSNPIEDKDA VTLTCEPETQ
351    DTTYLWWVNN QSLSVSSRLE LSNDNRTLTV FNIPRNDTTF YECETQNPVS
401    VRRSDPVTLN VLYGPDAPTI SPLNTPYRAG ENLNLSCHAA SNPAAQYSWF
451    VNGTFQQSTQ ELFIPNITVN NSGSYMCQAH NSATGLNRTT VTAITVYVEL
501    PKPYISSNNS NPIEDKDAVT LTCEPVAENT TYLWWVNNQS LSVSPRLQLS
551    NGNRILTLLS VTRNDTGPYE CGIQNSESAK RSDPVTLNVT YGPDTPIISP
601    PDLSYRSGAN LNLSCHSDSN PSPQYSWLIN GTLRQHTQVL FISKITSNNS
651    GAYACFVSNL ATGRNNSIVK NISVSSGDSA PGSSGLSARA TVGIIIGMLV
701    GVALM (SEQ ID NO:33.)
```

FIGURE 14C

```
  1    MGSPSAPLHR WCIPWQTLLL TASLLTFWNP PTTAQLTIES RPFNVAEGKE
 51    VLLLAHNVSQ NLFGYIWYKG ERVDASRRIG SCVIRTQQIT PGPAHSGRET
101    IDFNASLLIH NVTQSDTGSY TIQVIKEDLV NEEATGQFRV YPELPKPYIS
151    SNNSNPVEDK DAVALTCEPE TQDTTYLWWV NNQSLPVSPR LELSSDNRTL
201    TVFNIPRNDT TSYKCETQNP VSVRRSDPVT LNVLYGPDAP TISPLNTPYR
251    AGENLNLTCH AASNPTAQYF WFVNGTFQQS TQELFIPNIT VNNSGSYMCQ
301    AHNSATGLNR TTVTAITVYA ELPKPYITSN NSNPIEDKDA VTLTCEPETQ
351    DTTYLWWVNN QSLSVSSRLE LSNDNRTLTV FNIPRNDTTF YECETQNPVS
401    VRRSDPVTLN VLYGPDAPTI SPLNTPYRAG ENLNLSCHAA SNPAAQYFWF
451    VNGTFQQSTQ ELFIPNITVN NSGSYMCQAH NSATGLNRTT VTAITVYVEL
501    PKPYISSNNS NPIEDKDAVT LTCEPVAENT TYLWWVNNQS LSVSPRLQLS
551    NGNRILTLLS VTRNDTGPYE CGIQNSESAK RSDPVTLNVT YGPDTPIISP
601    PDLSYRSGAN LNLSCHSDSN PSPQYSWLIN GTLRQHTQVL FISKITSNNN
651    GAYACFVSNL ATGRNNSIVK NISVSSGDSA PGSSGLSARA TVGIIIGMLV
701    GVALM (SEQ ID NO:34)
```

FIGURE 14D

```
  1   MESPSAPPHR WCIPWQRLLL TASLLTFWNP PTTAKLTIES TPFNVAEGKE
 51   VLLLVHNLPQ HLFGYSWYKG ERVDGNRQII GYVIGTQQAT PGPAYSGREI
101   IYPNASLLIQ NIIQNDTGFY TLHVIKSDLV NEEATGQFRV YPELPKPSIS
151   SNNSKPVEDK DAVAFTCEPE TQDATYLWWV NNQSLPVSPR LQLSNGNRTL
201   TLFNVTRNDT ASYKCETQNP VSARRSDSVI LNVLYGPDAP TISPLNTSYR
251   SGENLNLSCH AASNPPAQYS WFVNGTFQQS TQELFIPNIT VNNSGSYTCQ
301   AHNSDTGLNR TTVTTITVYA EPPKPFITSN NSNPVEDEDA VALTCEPEIQ
351   NTTYLWWVNN QSLPVSPRLQ LSNDNRTLTL LSVTRNDVGP YECGIQNELS
401   VDHSDPVILN VLYGPDDPTI SPSYTYYRPG VNLSLSCHAA SNPPAQYSWL
451   IDGNIQQHTQ ELFISNITEK NSGLYTCQAN NSASGHSRTT VKTITVSAEL
501   PKPSISSNNS KPVEDKDAVA FTCEPEAQNT TYLWWVNGQS LPVSPRLQLS
551   NGNRTLTLFN VTRNDARAYV CGIQNSVSAN RSDPVTLDVL YGPDTPIISP
601   PDSSYLSGAN LNLSCHSASN PSPQYSWRIN GIPQQHTQVL FIAKITPNNN
651   GTYACFVSNL ATGRNNSIVK SITVSASGTS PGLSAGATVG IMIGVLVGVA
701   LI (SEQ ID NO:30)
```

FIGURE 14E

```
   1  ATGGGCAGCC CCAGCGCCCC CCTGCACCGC TGGTGCATCC CCTGGCAGAC
      CCTGCTGCTG ACCGCCAGCC TGCTGACCTT CTGGAACCCC CCCACCACCG
 101  CCCAGCTGAC CATCGAGAGC CGCCCCTTCA ACGTGGCCGA GGGCAAGGAG
      GTGCTGCTGC TGGCCCACAA CGTGAGCCAG AACCTGTTCG GCTACATCTG
 201  GTACAAGGGC GAGCGCGTGG ACGCCAGCCG CCGCATCGGC AGCTGCGTGA
      TCCGCACCCA GCAGATCACC CCCGGCCCCG CCCACAGCGG CCGCGAGACC
 301  ATCGACTTCA ACGCCAGCCT GCTGATCCAC AACGTGACCC AGAGCGACAC
      CGGCAGCTAC ACCATCCAGG TGATCAAGGA GGACCTGGTG AACGAGGAGG
 401  CCACCGGCCA GTTCCGCGTG TACCCCGAGC TGCCCAAGCC CTACATCAGC
      AGCAACAACA GCAACCCCGT GGAGGACAAG GACGCCGTGG CCCTGACCTG
 501  CGAGCCCGAG ACCCAGGACA CCACCTACCT GTGGTGGGTG AACAACCAGA
      GCCTGCCCGT GAGCCCCCGC CTGGAGCTGA GCAGCGACAA CCGCACCCTG
 601  ACCGTGTTCA ACATCCCCCG CAACGACACC ACCAGCTACA AGTGCGAGAC
      CCAGAACCCC GTGAGCGTGC GCCGCAGCGA CCCCGTGACC CTGAACGTGC
 701  TGTACGGCCC CGACGCCCCC ACCATCAGCC CCCTGAACAC CCCCTACCGC
      GCCGGCGAGA ACCTGAACCT GACCTGCCAC GCCGCCAGCA ACCCCACCGC
 801  CCAGTACTTC TGGTTCGTGA ACGGCACCTT CCAGCAGAGC ACCCAGGAGC
      TGTTCATCCC CAACATCACC GTGAACAACA GCGGCAGCTA CATGTGCCAG
 901  GCCCACAACA GCGCCACCGG CCTGAACCGC ACCACCGTGA CCGCCATCAC
      CGTGTACGCC GAGCTGCCCA AGCCCTACAT CACCAGCAAC AACAGCAACC
1001  CCATCGAGGA CAAGGACGCC GTGACCCTGA CCTGCGAGCC CGAGACCCAG
      GACACCACCT ACCTGTGGTG GGTGAACAAC CAGAGCCTGA GCGTGAGCAG
1101  CCGCCTGGAG CTGAGCAACG ACAACCGCAC CCTGACCGTG TTCAACATCC
      CCCGCAACGA CACCACCTTC TACGAGTGCG AGACCCAGAA CCCCGTGAGC
1201  GTGCGCCGCA GCGACCCCGT GACCCTGAAC GTGCTGTACG GCCCCGACGC
      CCCCACCATC AGCCCCCTGA ACACCCCCTA CCGCGCCGGC GAGAACCTGA
1301  ACCTGAGCTG CCACGCCGCC AGCAACCCCG CCGCCCAGTA CAGCTGGTTC
      GTGAACGGCA CCTTCCAGCA GAGCACCCAG GAGCTGTTCA TCCCCAACAT
1401  CACCGTGAAC AACAGCGGCA GCTACATGTG CCAGGCCCAC AACAGCGCCA
      CCGGCCTGAA CCGCACCACC GTGACCGCCA TCACCGTGTA CGTGGAGCTG
1501  CCCAAGCCCT ACATCAGCAG CAACAACAGC AACCCCATCG AGGACAAGGA
      CGCCGTGACC CTGACCTGCG AGCCCGTGGC CGAGAACACC ACCTACCTGT
1601  GGTGGGTGAA CAACCAGAGC CTGAGCGTGA GCCCCCGCCT GCAGCTGAGC
      AACGGCAACC GCATCCTGAC CCTGCTGAGC GTGACCCGCA ACGACACCGG
1701  CCCCTACGAG TGCGGCATCC AGAACAGCGA GAGCGCCAAG CGCAGCGACC
      CCGTGACCCT GAACGTGACC TACGGCCCCG ACACCCCCAT CATCAGCCCC
1801  CCCGACCTGA GCTACCGCAG CGGCGCCAAC CTGAACCTGA GCTGCCACAG
      CGACAGCAAC CCCAGCCCCC AGTACAGCTG GCTGATCAAC GGCACCCTGC
1901  GCCAGCACAC CCAGGTGCTG TTCATCAGCA AGATCACCAG CAACAACAGC
      GGCGCCTACG CCTGCTTCGT GAGCAACCTG GCCACCGGCC GCAACAACAG
2001  CATCGTGAAG AACATCAGCG TGAGCAGCGG CGACAGCTCT AGAAGCACCC
      CCATCCCATT CAGCTACAGC AAGAACCTGG ACTGCTGGGT GGACAACGAG
2101  GAGGACATCG ACGTGATCCT GAAGAAGAGC ACCATCCTGA ACCTGGACAT
      CAACAACGAC ATCATCAGCG ACATCAGCGG CTTCAACAGC AGCGTGATCA
2201  CCTACCCCGA CGCCCAGCTG GTGCCCGGCA TCAACGGCAA GGCCATCCAC
      CTGGTGAACA ACGAGAGCAG CGAGGTGATC GTGCACAAGG CCATGGACAT
2301  CGAGTACAAC GACATGTTCA ACAACTTCAC CGTGAGCTTC TGGCTGAGAG
      TGCCTAAGGT GAGCGCCAGC CACCTGGAGC AGTACGGCAC CAACGAGTAC
```

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2401    AGCATCATCA GCAGCATGAA GAAGCACAGC CTGAGCATCG GCAGCGGCTG
        GAGCGTGAGC CTGAAGGGCA ACAACCTCAT CTGGACCCTG AAGGATAGCG
2501    CCGGAGAGGT GAGACAGATC ACCTTCAGAG ACCTGCCCGA CAAGTTCAAT
        GCCTACCTGG CCAACAAGTG GGTGTTCATC ACCATCACCA ACGACAGACT
2601    GAGCAGCGCC AACCTGTACA TCAACGGCGT GCTCATGGGC AGCGCCGAGA
        TCACCGGCCT GGGCGCCATC AGAGAGGACA ACAACATCAC CCTGAAGCTG
2701    GACAGATGCA ACAACAACAA CCAGTACGTG AGCATCGACA AGTTCCGGAT
        CTTCTGCAAG GCCCTGAACC CCAAGGAGAT CGAGAAGCTG TACACCAGCT
2801    ACCTGAGCAT CACCTTCCTG AGAGACTTCT GGGGCAACCC CCTGAGATAC
        GACACCTAG (SEQ ID NO:35)
```

FIGURE 15A

```
  1   MGSPSAPLHR WCIPWQTLLL TASLLTFWNP PTTAQLTIES RPFNVAEGKE
 51   VLLLAHNVSQ NLFGYIWYKG ERVDASRRIG SCVIRTQQIT PGPAHSGRET
101   IDFNASLLIH NVTQSDTGSY TIQVIKEDLV NEEATGQFRV YPELPKPYIS
151   SNNSNPVEDK DAVALTCEPE TQDTTYLWWV NNQSLPVSPR LELSSDNRTL
201   TVFNIPRNDT TSYKCETQNP VSVRRSDPVT LNVLYGPDAP TISPLNTPYR
251   AGENLNLTCH AASNPTAQYF WFVNGTFQQS TQELFIPNIT VNNSGSYMCQ
301   AHNSATGLNR TTVTAITVYA ELPKPYITSN NSNPIEDKDA VTLTCEPETQ
351   DTTYLWWVNN QSLSVSSRLE LSNDNRTLTV FNIPRNDTTF YECETQNPVS
401   VRRSDPVTLN VLYGPDAPTI SPLNTPYRAG ENLNLSCHAA SNPAAQYSWF
451   VNGTFQQSTQ ELFIPNITVN NSGSYMCQAH NSATGLNRTT VTAITVYVEL
501   PKPYISSNNS NPIEDKDAVT LTCEPVAENT TYLWWVNNQS LSVSPRLQLS
551   NGNRILTLLS VTRNDTGPYE CGIQNSESAK RSDPVTLNVT YGPDTPIISP
601   PDLSYRSGAN LNLSCHSDSN PSPQYSWLIN GTLRQHTQVL FISKITSNNS
651   GAYACFVSNL ATGRNNSIVK NISVSSGDSS RSTPIPFSYS KNLDCWVDNE
701   EDIDVILKKS TILNLDINND IISDISGFNS SVITYPDAQL VPGINGKAIH
751   LVNNESSEVI VHKAMDIEYN DMFNNFTVSF WLRVPKVSAS HLEQYGTNEY
801   SIISSMKKHS LSIGSGWSVS LKGNNLIWTL KDSAGEVRQI TFRDLPDKFN
851   AYLANKWVFI TITNDRLSSA NLYINGVLMG SAEITGLGAI REDNNITLKL
901   DRCNNNNQYV SIDKFRIFCK ALNPKEIEKL YTSYLSITFL RDFWGNPLRY
951   DT* (SEQ ID NO:36)
```

FIGURE 15B

```
   1  ATGGGCAGCC CCAGCGCCCC CCTGCACCGC TGGTGCATCC CCTGGCAGAC
      CCTGCTGCTG ACCGCCAGCC TGCTGACCTT CTGGAACCCC CCCACCACCG
 101  CCCAGCTGAC CATCGAGAGC CGCCCCTTCA ACGTGGCCGA GGGCAAGGAG
      GTGCTGCTGC TGGCCCACAA CGTGAGCCAG AACCTGTTCG GCTACATCTG
 201  GTACAAGGGC GAGCGCGTGG ACGCCAGCCG CCGCATCGGC AGCTGCGTGA
      TCCGCACCCA GCAGATCACC CCCGGCCCCG CCCACAGCGG CCGCGAGACC
 301  ATCGACTTCA ACGCCAGCCT GCTGATCCAC AACGTGACCC AGAGCGACAC
      CGGCAGCTAC ACCATCCAGG TGATCAAGGA GGACCTGGTG AACGAGGAGG
 401  CCACCGGCCA GTTCCGCGTG TACCCCGAGC TGCCCAAGCC CTACATCAGC
      AGCAACAACA GCAACCCCGT GGAGGACAAG GACGCCGTGG CCCTGACCTG
 501  CGAGCCCGAG ACCCAGGACA CCACCTACCT GTGGTGGGTG AACAACCAGA
      GCCTGCCCGT GAGCCCCCGC CTGGAGCTGA GCAGCGACAA CCGCACCCTG
 601  ACCGTGTTCA ACATCCCCCG CAACGACACC ACCAGCTACA AGTGCGAGAC
      CCAGAACCCC GTGAGCGTGC GCCGCAGCGA CCCCGTGACC CTGAACGTGC
 701  TGTACGGCCC CGACGCCCCC ACCATCAGCC CCCTGAACAC CCCCTACCGC
      GCCGGCGAGA ACCTGAACCT GACCTGCCAC GCCGCCAGCA ACCCCACCGC
 801  CCAGTACTTC TGGTTCGTGA ACGGCACCTT CCAGCAGAGC ACCCAGGAGC
      TGTTCATCCC CAACATCACC GTGAACAACA GCGGCAGCTA CATGTGCCAG
 901  GCCCACAACA GCGCCACCGG CCTGAACCGC ACCACCGTGA CCGCCATCAC
      CGTGTACGCC GAGCTGCCCA AGCCCTACAT CACCAGCAAC AACAGCAACC
1001  CCATCGAGGA CAAGGACGCC GTGACCCTGA CCTGCGAGCC CGAGACCCAG
      GACACCACCT ACCTGTGGTG GGTGAACAAC CAGAGCCTGA GCGTGAGCAG
1101  CCGCCTGGAG CTGAGCAACG ACAACCGCAC CCTGACCGTG TTCAACATCC
      CCCGCAACGA CACCACCTTC TACGAGTGCG AGACCCAGAA CCCCGTGAGC
1201  GTGCGCCGCA GCGACCCCGT GACCCTGAAC GTGCTGTACG GCCCCGACGC
      CCCCACCATC AGCCCCCTGA ACACCCCCTA CCGCGCCGGC GAGAACCTGA
1301  ACCTGAGCTG CCACGCCGCC AGCAACCCCG CCGCCCAGTA CAGCTGGTTC
      GTGAACGGCA CCTTCCAGCA GAGCACCCAG GAGCTGTTCA TCCCCAACAT
1401  CACCGTGAAC AACAGCGGCA GCTACATGTG CCAGGCCCAC AACAGCGCCA
      CCGGCCTGAA CCGCACCACC GTGACCGCCA TCACCGTGTA CGTGGAGCTG
1501  CCCAAGCCCT ACATCAGCAG CAACAACAGC AACCCCATCG AGGACAAGGA
      CGCCGTGACC CTGACCTGCG AGCCCGTGGC CGAGAACACC ACCTACCTGT
1601  GGTGGGTGAA CAACCAGAGC CTGAGCGTGA GCCCCCGCCT GCAGCTGAGC
      AACGGCAACC GCATCCTGAC CCTGCTGAGC GTGACCCGCA ACGACACCGG
1701  CCCCTACGAG TGCGGCATCC AGAACAGCGA GAGCGCCAAG CGCAGCGACC
      CCGTGACCCT GAACGTGACC TACGGCCCCG ACACCCCCAT CATCAGCCCC
1801  CCCGACCTGA GCTACCGCAG CGGCGCCAAC CTGAACCTGA GCTGCCACAG
      CGACAGCAAC CCCAGCCCCC AGTACAGCTG GCTGATCAAC GGCACCCTGC
1901  GCCAGCACAC CCAGGTGCTG TTCATCAGCA AGATCACCAG CAACAACAGC
      GGCGCCTACG CCTGCTTCGT GAGCAACCTG GCCACCGGCC GCAACAACAG
2001  CATCGTGAAG AACATCAGCG TGAGCAGCGG CGACAGCTCT AGAACCCCTC
      AGAACATCAC CGATCTGTGC GCCGAGTACC ACAACACCCA GATCTACACC
2101  CTGAACGACA AGATCTTCAG CTACACCGAG AGCCTGGCCG GCAAGAGAGA
      GATGGCCATC ATCACCTTCA AGAACGGCGC CATCTTCCAG GTGGAGGTGC
2201  CCGGCAGCCA GCACATCGAC AGCCAGAAGA AGGCCATCGA GCGGATGAAG
      GACACCCTGC GGATCGCCTA CCTCACCGAG GCCAAGGTGG AGAAGCTGTG
2301  CGTGTGGAAC AACAAGACCC CTCACGCCAT CGCCGCCATC AGCATGGCCA
      ATTGATAAG (SEQ ID NO:37)
```

FIGURE 16A

```
  1   MGSPSAPLHR WCIPWQTLLL TASLLTFWNP PTTAQLTIES RPFNVAEGKE
 51   VLLLAHNVSQ NLFGYIWYKG ERVDASRRIG SCVIRTQQIT PGPAHSGRET
101   IDFNASLLIH NVTQSDTGSY TIQVIKEDLV NEEATGQFRV YPELPKPYIS
151   SNNSNPVEDK DAVALTCEPE TQDTTYLWWV NNQSLPVSPR LELSSDNRTL
201   TVFNIPRNDT TSYKCETQNP VSVRRSDPVT LNVLYGPDAP TISPLNTPYR
251   AGENLNLTCH AASNPTAQYF WFVNGTFQQS TQELFIPNIT VNNSGSYMCQ
301   AHNSATGLNR TTVTAITVYA ELPKPYITSN NSNPIEDKDA VTLTCEPETQ
351   DTTYLWWVNN QSLSVSSRLE LSNDNRTLTV FNIPRNDTTF YECETQNPVS
401   VRRSDPVTLN VLYGPDAPTI SPLNTPYRAG ENLNLSCHAA SNPAAQYSWF
451   VNGTFQQSTQ ELFIPNITVN NSGSYMCQAH NSATGLNRTT VTAITVYVEL
501   PKPYISSNNS NPIEDKDAVT LTCEPVAENT TYLWWVNNQS LSVSPRLQLS
551   NGNRILTLLS VTRNDTGPYE CGIQNSESAK RSDPVTLNVT YGPDTPIISP
601   PDLSYRSGAN LNLSCHSDSN PSPQYSWLIN GTLRQHTQVL FISKITSNNS
651   GAYACFVSNL ATGRNNSIVK NISVSSGDSS RTPQNITDLC AEYHNTQIYT
701   LNDKIFSYTE SLAGKREMAI ITFKNGAIFQ VEVPGSQHID SQKKAIERMK
751   DTLRIAYLTE AKVEKLCVWN NKTPHAIAAI SMAN** (SEQ ID NO:38)
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FIGURE 16B